**Glenda Wiles**

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**From:** bjhoy@localnet.com
**Sent:** Wednesday, July 3, 2019 2:55 PM
**To:** Ravalli County Commissioners Office
**Subject:** Attachments
**Attachments:** LC-ESIMSMS_analysis_of_neonicotinoids_in_urine_of_ (1).pdf; glyphosate_air_IL13 _inflammation_2014.pdf; cdc_45178_DS1.pdf

Hi Glenda,

Sorry, I thought I attached the study about newborn babies and Imidacloprid. It is first below.

There is also a study about glyphosate, which strongly indicate it causes damaged lungs, which I am attaching. I have been told that studies about glyphosate that find it does very bad things at very low doses is a "witch hunt" against Roundup and Monsanto.

I also just received a study that says that breathing pesticides causes farmers to wheeze (attached last). Pesticides cause me to wheeze, so I can sympathize with them, but not much since they spray the stuff, causing their own exposure. I don't use any pesticides because I am very allergic to them.

Thank you for telling me I forgot to attach the study.

Happy Fourth of July,
Judy

PLOS ONE

RESEARCH ARTICLE

# LC-ESI/MS/MS analysis of neonicotinoids in urine of very low birth weight infants at birth

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## Abstract

### Objectives

Neonicotinoid insecticides are widely used systemic pesticides with nicotinic acetylcholine receptor agonist activity that are a concern as environmental pollutants. Neonicotinoids in humans and the environment have been widely reported, but few studies have examined their presence in fetuses and newborns. The objective of this study is to determine exposure to neonicotinoids and metabolites in very low birth weight (VLBW) infants.

### Methods

An analytical method for seven neonicotinoids and one neonicotinoid metabolite, *N*-desmethylacetamiprid (DMAP), in human urine using LC-ESI/MS/MS was developed. This method was used for analysis of 57 urine samples collected within 48 hours after birth from VLBW infants of gestational age 23–34 weeks (male/female = 36/21, small for gestational age (SGA)/appropriate gestational age (AGA) = 6/51) who were admitted to the neonatal intensive care unit of Dokkyo Hospital from January 2009 to December 2010. Sixty-five samples collected on postnatal day 14 (M/F = 37/22, SGA/AGA = 7/52) were also analyzed.

### Results

DMAP, a metabolite of acetamiprid, was detected in 14 urine samples collected at birth (24.6%, median level 0.048 ppb) and in 7 samples collected on postnatal day 14 (11.9%, median level 0.09 ppb). The urinary DMAP detection rate and level were higher in SGA than in AGA infants (both $p<0.05$). There were no correlations between the DMAP level and infant physique indexes (length, height, and head circumference SD scores).

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**Competing interests:** The authors have declared that no competing interests exist.

## Conclusion

These results provide the first evidence worldwide of neonicotinoid exposure in newborn babies in the early phase after birth. The findings suggest a need to examine potential neurodevelopmental toxicity of neonicotinoids and metabolites in human fetuses.

## Introduction

Neonicotinoid insecticides (neonicotinoids) are neurotoxicants with nicotinic acetylcholine receptor (nAChR) modulator action [1–3]. Neonicotinoids were first introduced on the market in the mid-1990s, and now are the most widely used class of insecticides worldwide, both for seed dressings of crops (e.g. maize, oil-seed rape, cotton, and soybeans) and for spraying on rice paddies, fruits, vegetables, tea leaves, cocoa, and coffee beans [3, 4]. In Japan, seven neonicotinoids were registered as pesticides up to 2002, with 70.3 tons of imidacloprid, 53.8 tons of acetemiprid, 8.0 tons of nitenpyram, 21.4 tons of thiacloprid, 34.4 tons of thiamethoxam, 64.2 tons of clothianidin, and 156.8 tons of dinotefuran shipped in 2009 [5,6]. Three more insecticides with nAChR modulator action have recently been registered: flupyradifurone in 2015, and sulfoxaflor and triflumezopyrim in 2018 [7]. These three molecules are also considered to be neonicotinoids because of their similarity in molecular structure and neuronal effects to those of the original seven neonicotinoids [8].

Since the mid-2000s, many studies have shown that neonicotinoids may have negative effects on non-targeted invertebrates, in particular on honeybees and bumblebees [1,4,8,9–13]. This evidence has led to prohibition of outside use of three neonicotinoids, imidacloprid, clothianidin and thiamethoxam, in the EU since 2013 [14–17] and a total ban on outside use of imidacloprid, clothianidin, thiamethoxam, thiacloprid, and acetamiprid in France in 2019 [18].

Neonicotinoids may also have negative effects on vertebrates [13, 19], including wild birds [20], bats [21], and white-tailed deer [22]. Recent *in vitro* studies have revealed multiple toxicity of neonicotinoids at a low dose, including neurotoxicity of imidacloprid at 0.77 mg/L [23], immunotoxicity of clothianidin at 0.1 mg/L [24], endocrine toxicity of imidacloprid at 0.03 mg/L and thiacloprid at 0.08 mg/L [25], and genotoxicity caused by oxidative stress [26]. A few *in vivo* studies have shown neurodevelopmental toxicity in rodents by imidacloprid 0.5 mg/kg/day, and acetamiprid 1 mg/kg/day, and neurotoxicity with clothianidin 10 mg/kg/day [27–29]. These levels are the same or lower than the no-observed-adverse-effect levels (NOAELs) of 5.7 mg/kg/day for imidacloprid, 7.1 mg/kg/day for acetamiprid, and 9.7 mg/kg/day for clothianidin [30–32]. Several reports suggest that subacute and chronic exposure to neonicotinoids such as acetamiprid and thiamethoxam may be toxic in humans [33,34], and acute high dose exposure of imidacloprid [35], acetamiprid [36, 37], and thiacloprid [38,39] can be lethal. Neonicotinoids are well absorbed by humans after oral intake and are mainly excreted in urine [40–42]. These molecules cross the human blood brain barrier [43], and some neonicotinoids have toxic metabolites, such as desnitroimidacloprid, which has a mammalian nAChR agonistic activity that is as high as that of nicotine [44].

Current reports of neonicotinoid food contamination at less than the maximum residual dose are increasing. Japanese non-organic green tea leaves are contaminated by dinotefuran with imidacloprid, acetamiprid, clothianidin, thiacloprid and thiamethoxam [45]. In the EU, acetamiprid was detected in 10% of apples, imidacloprid in 15.8% of lettuces, and thiacloprid in 11.4% of strawberries [46]. In the US, acetamiprid was detected in 13.4% of fruits and

imidacloprid in 19.9% of vegetables in 1999–2015 [47]. A particularly toxic neonicotinoid metabolite, desnitro-imidacloprid, has been detected in drinking water in the US [48]. Unlike most other pesticides, neonicotinoids cannot be washed off food prior to consumption due to the characteristics of the plant [47].

Frequent detection of neonicotinoids and their metabolites in urine and hair have been reported for the general population [42,49–52], but this has not been investigated in fetuses and newborn babies, despite their potentially high sensitivity to these chemicals [53]. Developing cerebral vessels in infants are more fragile than those in adults and more vulnerable to drugs, toxins, and pathological conditions, which may cause cerebral damage and subsequent neurological disorders [54]. Many adult functions, including effective tight junctions, are not developed in the embryonic brain and some transporters are more active during development than in adults [55,56].

In Japan in 2009, the incidence of low-birth-weight (LBW) infants (<2500 g at birth) was 9.6%, and that of very (V)LBW infants (<1500 g) was 0.74% [57]. LBW infants are classified as small for gestational age (SGA), indicating those who are smaller in size, with weight below the $10^{th}$ percentile for gestational age; or appropriate for gestational age (AGA), for those who are appropriate in size, with weight and head circumference in the range from the $10^{th}$ to $90^{th}$ percentile. In general, neurological development in SGA infants is worse than that in AGA infants [58]. In addition to body weight, head circumference is used as an index of development, and the head circumference SD score can be calculated by the lambda-mu-sigma method using LMS chart-maker. This score is the international standard for newborn size for each gestational age based on data from the Newborn Cross-Sectional Study, which conforms at the population and individual levels to the prescriptive approach used in the WHO Multicentre Growth Reference Study [59]. A low head circumference SD score is related to neurodevelopment delay [58].

In this study, we developed an analytical method for seven neonicotinoids and one neonicotinoid metabolite in human urine. Then we explored exposure to neonicotinoids and metabolites in VLBW infants born in 2009–2010 in the early stage after birth to examine whether neonicotinoids can be transferred to fetuses. These infants are not usually fed with milk for 48 hours at birth. The relationships of detection of neonicotinoids with body weight and head circumference SD scores were also examined.

## Subjects and methods

### Subjects and sample collection

The subjects were infants born at a gestational age of 23 to 32 weeks and a birth weight of 500–1,500 g who were admitted to the NICU of Dokkyo Medical University Hospital from January 2009 to December 2010. Infants with chromosomal abnormalities, external deformities and life-threatening diseases were excluded. After obtaining approval from the ethics committee of Dokkyo Medical University (approval no. 25042) and informed consent from the infants' parents, urine samples were collected on postnatal days (PNDs) 1 to 2 (within 48 h after birth) and PND 14 using cotton balls or a urine sampling bag, and the samples were stored at -80˚C. The cotton ball was applied to the absorbent core in the diaper and collected after it was immersed in urine. The current study was performed using urine samples collected from these infants for a previous study. We obtained new approval from the ethics committee of Dokkyo Medical University (approval no.29008) and gave an explanation to the infants' parents by posting a notice and through an opt-out method, which was also approved by the ethics committee of Dokkyo Medical University.

## Chemicals

Acetamiprid, dinotefuran, imidacloprid, nitenpyram and thiacloprid were purchased from Kanto Chemical Corp. (Tokyo, Japan). Clothianidin, clothianidin-d3, dinotefuran-d3, imidacloprid-d4, thiacloprid-d4, thiamethoxam-d4, and *N*-desmethylacetamiprid (DMAP) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hydroxyimidacloprid was purchased from Hypha Discovery (Slough, UK). Acetamiprid-d6 and nitenpyram-d3 were purchased from Hayashi Pure Chemical Ind. (Osaka, Japan). Thiamethoxam was purchased from Dr. Ehrenstorfer. Acetonitrile, dichloromethane formic acid, ammonium acetate and distilled water were all HPLC grade and were purchased from Kanto Chemical.

**Urine sample preparation and analysis.** Urine was thawed, stirred, and allowed to stand for some time and the supernatant was used. Purification of urine was performed by solid phase extraction (SPE). A volume of 100 μL of internal standard mixture (each 10 ppb) was added to 100 μL of each urine sample, and then 2800 μL of distilled water was added to the sample. Two types of SPE cartridges were used for purification: an InertSep Pharma SPE column (60 mg/3 ml) (GL Science, Tokyo, Japan) pre-conditioned with 3 mL of an acetonitrile/dichloromethane (1/1) mixture followed by 3 ml of distilled water; and an InertSep PSA SPE column (100 mg/1ml) (GL Science) pre-conditioned with 1 mL of the acetonitrile/dichloromethane (1/1) mixture. Prepared samples were loaded on the pre-conditioned InertSep Pharma and washed with 0.5 mL of distilled water. The InertSep Pharma (top) was combined with the InertSep PSA (bottom) and 3 ml of the acetonitrile/dichloromethane (1/ 1) mixture were used to elute the target chemicals. After concentrating and dry-solidifying with a centrifugal concentrator (CVE-200D with UT-2000, Eyela, Tokyo, Japan), the samples were reconstituted with 100 μL of 3% methanol in distilled water and transferred to vials for analysis. Seven neonicotinoids and DMAP were analyzed in each sample. Recovery rates and LOQs are shown in Table 1.

A LC-ESI/MS/MS system (Agilent 6495B, Agilent Technologies, Santa Clara, CA, USA) equipped with a Kinetex Biphenyl column (2.1 mm ID × 100 mm, ϕ2.6 μm, Phenomenex, Torrance, CA, USA) was used for sample analysis. Solvents A and B used for HPLC analysis were 0.1% formic acid + 10 mM ammonium acetate in aqueous solution and 0.1% formic acid + 10 mM ammonium acetate in methanol, respectively. The gradient was programmed as: $t = 0$ to 1 min: 5% B, $t = 6$ min: 95% B, $t = 6$ to 8 min: 95% B. The column oven temperature and flow rate were 60˚C and 0.5 ml/min, respectively. For mass spectrometry, multiple reaction monitoring (MRM) was programmed (Table 1). The recovery rate of each neonicotinoid and its metabolites ranged from 80% (acetamiprid) to 117% (thiamethoxam). The reproducibility of the analysis system was confirmed in the same or plural analyses, with a relative standard deviation (RSD) of 10% for all the compounds.

**Quantitation of neonicotinoids and their metabolites.** Seven neonicotinoids and DMAP (Table 1) were analyzed in each sample. Six compounds were used as internal standards. The precursor and product ions are shown in Table 1. Quantification of the neonicotinoids and metabolites was carried out by the internal standard method. Five calibration points were set at 0.5, 1.25, 2.5, 3.75 and 5 ppb, whereas the internal standard was used to 5 ppb at all calibration points.

**Quality control and quality assurance.** A mixture of six deuterium-labeled neonicotinoids was spiked into samples as an internal standard prior to sample preparation and extraction. Quantitation was performed using five calibration points and the average coefficients of determination ($r^2$) for the calibration curves were $\geq$0.995. The analytical method was checked for precision and accuracy. Limits of detection (LODs) were calculated based on $3SD/S$ ($SD$ is the standard deviation of the response of seven replicate standard solution measurements and

**Table 1. Properties of target neonicotinoids and metabolites.**

| Neonicotinoids | MRM[a] (*m/z*) | RT[b] (min) | Recovery rate (%) | LOQ[c] (ng/ml) |
|---|---|---|---|---|
| Dinotefuran | 203.00>129.10 | 8.2 | 92.6 ±2.8 | 0.125 |
| Nitenpyram | 271.00>126.05 | 8.9 | 88.6 ±4.6 | 0.5 |
| Thiamethoxam | 291.90>211.00 | 14.0 | 116.7 ±7.9 | 0.125 |
| *N*-Desmethylacetamiprid (DMAP) | 208.90>126.05 | 15.2 | 87.6 ±5.4 | 0.05 |
| Clothianidin | 249.90>132.05 | 16.1 | 91.8 ±3.7 | 0.125 |
| Acetamiprid | 223.00>126.00 | 16.2 | 80.2 ±2.9 | 0.05 |
| Imidacloprid | 256.00>209.05 | 17.3 | 87.0 ±2.7 | 0.5 |
| Thiacloprid | 252.90>126.05 | 19.1 | 92.9 ±1.8 | 0.05 |

[a] MRM: multiple reaction monitoring;

[b]RT: Retention time;

[c]LOQ: limit of quantification

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*S* is the slope of the calibration curve). Recovery rates and LOQs (ng/mL) of the analytes are given in Table 1.

## Statistical analysis

IBM SPSS Statistics 23 was used for statistical analysis. A Fisher exact test was used to compare the DMAP detection rates. A Wilcoxon rank sum test for non-parametric data was used to compare DMAP concentrations. Spearman rank correlation coefficient analysis was used to compare the DMAP level and infant physique index (length SD score, height SD score, and head circumstance SD score). The significance level was set at $P = 0.05$.

# Results

Fourteen of the 130 urine samples collected from 65 subjects could not be analyzed due to insufficient volume, and thus, the final analysis included 116 samples: 57 collected on PND 1–2 (within 48 h after birth) and 59 on PND 14. The background of the subjects, including physical status, is shown in Table 2.

**Table 2. Characteristics of infants on postnatal days (PNDS) 1–2 and 14.**

| Item | PND 1–2 | PND 14 | P |
|---|---|---|---|
| Number of samples | 57 | 59 | |
| Gestational age (weeks) | 27 (28, 23–34) | 27 (28, 23–34) | 0.84 |
| Sex, male | 36 (63%) | 37 (63%) | 0.92 |
| Birth weight (g) | 1012 (982,515–1474) | 967 (926,515–1474) | 0.69 |
| Birth weight SD score | -0.6 (-0.6,-3.1–2.0) | -0.6 (0.1,-3.3–2.0) | 0.75 |
| Birth length (cm) | 35.1 (35, 28–41) | 34.7 (35, 28–41) | 0.99 |
| Birth length SD score | -0.4 (-0.4,-2.6–1.7) | -0.4 (-0.4,-2.6–1.7) | 0.77 |
| Head circumference (cm) | 25.3 (25.5,19.8–29.5) | 25.0 (24.5,19.8–29.5) | 0.89 |
| Head circumference SD score | 0.1 (0.2,-1.8–1.4) | 0.1(0.3,-1.8–1.4) | 0.88 |
| Small for gestational age | 6 (11%) | 8 (14%) | 0.32 |
| Apgar score 5 min | 7.6 (8, 1–10) | 7.5(7, 1–10) | 0.75 |
| Cesarean section | 35 (61%) | 38 (64%) | 0.51 |

Data are shown as a mean (median, range) or as n (%)

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**Table 3. Detection of DMAP in urine of VLBW infants on postnatal days (PNDS) 1–2 and 14.**

| Item | PND 1–2 | PND 14 | p |
|---|---|---|---|
| Detection rate (%) (number of samples) | 24.6 (14/57) | 11.9 (7/59) | 0.09 |
| Mean concentration (ppb) (median, range) | 0.11 (0.048, 0.01–0.68) | 0.13 (0.09, 0.01–0.47) | 0.09 |

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**Table 4. Differences in detection rates and levels of DMAP in SGA and AGA infants.**

| Item | SGA[a] | AGA[b] | p |
|---|---|---|---|
| Detection rate (%) (number of samples) | 42.9 (6/14) | 14.7 (15/102) | 0.005 |
| Mean concentration (ppb) (median, range) | 0.04 (0, 0–0.3) | 0.02 (0, 0–0.68) | 0.004 |

[a]SGA: small for gestational age;

[b]AGA: appropriate for gestational age

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**Table 5. Relationships of detection of DMAP on PND 1–2 and head circumference SD score.**

| Item | Head circumference SD score | | p |
|---|---|---|---|
| | Positive | Negative | |
| Detection rate (%) (number of samples) | 16.7 (6/36) | 38.1 (8/21) | 0.07 * |
| Mean concentration (ppb) (median, range) | 0.025 (0, 0–0.68) | 0.032 (0, 0–0.30) | 0.07† |

*p by $\chi^2$ test.

†p by Wilcoxon rank sum test.

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Dinotefuran was detected in one sample and one of the 20 neonicotinoid metabolites, DMAP, was also detected. The concentration of dinotefuran was 0.4 ppb (PND 1–2). DMAP was detected in 14/57 PND 1–2 samples (24.6%) (median level 0.048 ppb, range: 0.01–0.68 ppb), and in 7/59 PND 14 samples (11.9%) (median level 0.09 ppb, range: 0.01–0.47 ppb), with no significant difference in the detection rate ($p = 0.09$) or level ($p = 0.09$) between PND 1–2 and PND 14 samples (Table 3).

The DMAP detection rate was significantly higher in SGA infants than in AGA infants (42.9% vs. 14.7%, $p<0.05$). The mean DMAP level was similarly significantly higher in SGA infants (0.04 vs. 0.02 ppb, $p<0.05$) (Table 4). There was no significant difference in DMAP detection rates on PND 1–2 for infants with positive or negative head circumference SD scores ($p = 0.07$) (Table 5). In infants in whom DMAP was detected on PND 1–2, there were weak negative correlations between DMAP levels and birth weight SD scores ($\rho = -0.37$, $p = 0.19$), birth length SD scores ($\rho = -0.36$, $p = 0.20$), and birth head circumference SD scores ($\rho = -0.23$, $p = 0.43$), but none of these relationships were significant.

## Discussion

This is the first report worldwide to suggest that DMAP, a toxic metabolite of acetamiprid, may transfer to fetuses at a high rate. The oral median lethal dose ($LD_{50}$) of DMAP is 1,843 mg/kg in female rats [31]. DMAP is the most frequently detected neonicotinoid metabolite in the general Japanese population by ppb level [42], and Marfo et al. reported that DMAP is often detected in patients with symptoms of recent memory loss and finger tremor [34].

DMAP might be transferred via the placenta and accumulate in the fetus, because it was frequently detected in urine collected on PND 1–2 and the level did not increase significantly on PND 14. Acetamiprid is rapidly metabolized to DMAP after oral administration in healthy

male adults [42]. Its urinary excretion half-life is 1.65 days [38]. Continuous maternal intake of acetamiprid may cause high DMAP levels in maternal blood and DMAP contamination in the fetus. There are two possible reasons why DMAP was detected at a higher rate in SGA infants. First, body composition differs between SGA and AGA infants: % body fat is lower in SGA infants and the brain volume is relatively large. Assuming that DMAP accumulates via nAChRs in the brain, more neonicotinoids may accumulate in SGA infants. A second reason is that DMAP might inhibit growth by affecting neurological development of the fetus.

The parent compound of DMAP, acetamiprid, is a common neonicotinoid in Japan that is used for a wide range of plant protection, including fruits, vegetables, tea leaves, rice paddies, turf, ornamental flowers, and pine trees. The oral $LD_{50}$ of acetamiprid is 146 mg/kg in female rats [31]. Fatal cases of human acute intoxication have also been reported [36]. In acute intoxication by acetamiprid, nicotinic symptoms including neuronal symptoms are observed [37]. Acetamiprid has some lipophilicity (Log $P_{ow}$ is 0.8) and is not ionized at physiological pH [31], which suggests that it may be retained in the human body, even if its receptor action is weak. There is also some evidence to suggest that acetamiprid is toxic for neurological development [27, 59–61]. It has yet to be clarified whether neonicotinoids have neurological toxicity in infants, but the safety of acetamiprid should be reviewed based on the possibility that neonicotinoids may transfer to and accumulate in fetuses at a high rate.

The limitations of this study include the small number of subjects, investigation in one region in Japan, the inclusion of VLBW infants born prematurely rather than term newborns, and the lack of examination of pesticides other than neonicotinoids. However, it is likely that exposure to neonicotinoids observed in infants born prematurely will be similar in term newborns because they experience a similar period of exposure. Further studies are needed in a larger number of subjects in various regions, but similar results are likely because the use and environmental detection rates of neonicotinoids have increased worldwide.

## Conclusion

This report provides the first evidence worldwide showing that *N*-desmethylacetamiprid (DMAP), a metabolite of acetamiprid, can be transferred to fetuses. DMAP levels were also significantly higher in SGA infants than in AGA infants. The fetal and neonatal periods are extremely important for neurological development, and further studies are needed with regard to the safety of acetamiprid due to transfer and accumulation of its metabolite in the womb.

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## Author Contributions

**Conceptualization:** Go Ichikawa.

**Data curation:** Yoshinori Ikenaka.

**Formal analysis:** Go Ichikawa, Yoshinori Ikenaka.

**Funding acquisition:** Yoshinori Ikenaka, Mayumi Ishizuka, Kumiko Taira.

**Investigation:** Go Ichikawa.

**Methodology:** Takahiro Ichise, Shouta M. M. Nakayama.

**Project administration:** Go Ichikawa.

**Resources:** Ryota Kuribayashi.

**Supervision:** Kumiko Taira, Toshimi Sairenchi, Gen Kobashi, Shigemi Yoshihara.

**Visualization:** Go Ichikawa.

**Writing – original draft:** Go Ichikawa.

**Writing – review & editing:** Yoshinori Ikenaka, Kumiko Taira, Kazutoshi Fujioka, Jean-Marc Bonmatin.

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# Glyphosate–rich air samples induce IL–33, TSLP and generate IL–13 dependent airway inflammation

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## Abstract

Several low weight molecules have often been implicated in the induction of occupational asthma. Glyphosate, a small molecule herbicide, is widely used in the world. There is a controversy regarding a role of glyphosate in developing asthma and rhinitis among farmers, the mechanism of which is unexplored. The aim of this study was to explore the mechanisms of glyphosate induced pulmonary pathology by utilizing murine models and real environmental samples. C57BL/6, TLR4−/−, and IL-13−/− mice inhaled extracts of glyphosate-rich air samples collected on farms during spraying of herbicides or inhaled different doses of glyphosate and ovalbumin. The cellular response, humoral response, and lung function of exposed mice were evaluated. Exposure to glyphosate-rich air samples as well as glyphosate alone to the lungs increased: eosinophil and neutrophil counts, mast cell degranulation, and production of IL-33, TSLP, IL-13, and IL-5. In contrast, *in vivo* systemic IL-4 production was not increased. Co-administration of ovalbumin with glyphosate did not substantially change the inflammatory immune response. However, IL-13-deficiency resulted in diminished inflammatory response but did not have a significant effect on airway resistance upon methacholine challenge after 7 or 21 days of glyphosate exposure. Glyphosate-rich farm air samples as well as glyphosate alone were found to induce pulmonary IL-13-dependent inflammation and promote Th2 type cytokines, but not IL-4 for glyphosate alone.



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**Conflicts of interest**

The authors declare that there are no conflicts of interest.

This study, for the first time, provides evidence for the mechanism of glyphosate-induced occupational lung disease.

**Keywords**

Occupational health; lung disease; asthma; pesticide; innate immunity; immunotoxicology

## 1. Introduction

Many low–molecular weight chemicals including some pesticides and herbicides are capable of inducing occupational asthma (Henneberger et al., 2014). Glyphosate [(N-phosphonomethyl)glycine] is one of the most commonly used broad spectrum nonselective herbicides in the world. Approximately 83,000 tons of it was applied to agricultural fields and approximately 3000 tons was applied to the lawns and garden areas of homes in the United States (USA EPA, 2007).

Since glyphosate was brought to market in the 1970's as the active ingredient in the formulation of Roundup®, several animal studies have investigated the toxicity of glyphosate when administered by intravenous, oral, intraperitoneal, dermal, and ocular routes (Tai et al., 1990; Agriculture Canada, 1991; Cox, 1995). Its gastrointestinal toxicity to humans was also documented (Sawada et al., 1988; Talbot et al., 1991; Tominack et al., 1991; Menkes et al., 1991; Temple et al., 1992). According to the SAR (structure-activity relationships) model of Jarvis et al. (2005), the hazard index value of glyphosate is 0.6257, which evidently supports the hazardous nature of glyphosate and its possible role in inducing asthmatic symptoms. However, the inhalational effects of glyphosate particularly its effect on development of asthma was not entirely explored.

Because experimental asthma has been largely studied using various proteins as disease mediators, our understanding of asthma pathogenesis relies heavily on adaptive immune responses. The understanding of the induction of allergic pathology caused by small molecules like glyphosate is challenging due to a fundamentally distinct immune response that may not be obtainable like adaptive immune responses from environmental allergens of high molecular weights. Given that innate immune responses to stimuli are specific to the anatomic site involved, those animal studies that administered glyphosate to the airway would be best suited to provide insight into the pathogenesis of airway disease produced in agricultural workers. To our knowledge, the inhalational hazards of glyphosate have been studied experimentally by two groups (US EPA, 1982; Martinez et al., 1990). It was shown that glyphosate inhalation caused wheezing, reduced activity, and dark nasal discharge even at low exposure levels in rats. How these small molecules contribute to the development of these phenotypes remains a mystery. We hypothesized that exposure to air pollutant containing herbicides, endotoxin or other environmental contaminants induces airway inflammation by activation of the innate immune system through pattern recognizing innate pro–cytokines that contribute to the airway pathology. Here we report the exploration of the mechanism behind the airway inflammation caused by agricultural air samples containing

glyphosate, endotoxin, and other environmental contaminants as well as reagent–grade glyphosate delivered at low and high doses in the presence or absence of exogenous antigen.

## 2. Material s and methods

### *2.1.* Mice

C57BL/6 female (6–9 weeks) mice were purchased from Jackson Laboratory (Sacramento, CA). TLR4−/− mice (backcrossed 10 generations) were received from Cincinnati Children's Hospital Medical Center (CCHMC). Both strains were subsequently bred in house. Female mice of wild type and IL–13−/− BALB/c background were received from the laboratory of Dr. Fred Finkelman, CCHMC. Mice were housed in individually ventilated cages in a pathogen free facility at the Department of Environmental Health, University of Cincinnati (UC) following the UC Institutional Animal Care and Use Committee (IACUC) guidelines and all experiments were conducted following a UC IACUC–approved protocol.

### *2.2.* Antibodies and reagents

We purchased the following antibodies for flow cytometry: Ly–6G (Gr–1) eFluor® 450 (RB6–8C5; Isotype Rat IgG2b) from eBioscience (San Diego, CA). CD16/CD32 (2.4G2; Isotype Rat IgG2b) and SiglecF–PE (E50–2440; Isotype Rat IgG2a) were purchased from BD PharMingen (San Jose, CA). A kit for measuring serum levels of MMCP1 was purchased from eBioscience.

### *2.3.* Collection of farm air samples during summer pesticide spray seasons

Air samples were collected by three sets of total inhalable aerosol samplers (Button Inhalable Aerosol Sampler, SKC Inc., Eighty Four, PA) operated in parallel on three farms in Butler County, Ohio during summer glyphosate spray seasons. Samplers were installed at 1.5 m height at the edge of the field downwind from the spraying locations (sizes: approx. 5000–10000 $m^2$). The sampling period was approximately 24 hours starting from glyphosate spraying and air samples were collected at a flow rate of approximately 4 L/min on glass fiber filters. The filters from one set of samplers containing aerosolized glyphosate were eluted using PBS and the suspensions were filtered. A stock solution was prepared by pooling the samples collected from three farms (from now on referred as 'Real Env') and used for intranasal treatment of mice. The filters from the other two sets of samplers were analyzed for glyphosate and endotoxin to estimate the levels of glyphosate and endotoxin in 'Real Env' samples.

### *2.4.* Analysis of glyphosate in filter extracts

Glyphosate residues from filters were extracted using $KH_2PO_4$ buffer /1M NaOH in an automatic shaker followed by freeze drying. The freeze dried samples were dissolved with deionized water and filtered through 0.45 μM Millipore filter. Glyphosate levels in the suspensions were determined by Abraxis ELISA Kit at 450 nm. The average amount of glyphosate per filter was 17.33 μg, which correspond to average airborne concentration of 22.59 $ng/m^3$.

### 2.5. Analysis of endotoxin in filter extracts

Endotoxin in filter extracts were analyzed using the Limulus Amebocyte Lysate assay (Pyrochrome LAL; Associates of Cape Cod Inc, Falmouth, MA), as described previously (Adhikari et al., 2009; 2010). The samples were spiked with endotoxin standard of 0.50 EU/ml to assure that there was no inhibition or enhancement between the filter extracts and the reagents. The average amount of endotoxin per filter was 24.49 EU, which correspond to average airborne concentration of 4.87 EU/$m^3$.

### 2.6. Treatment of mice with farm-derived air samples, glyphosate and sensitization with OVA

PBS suspended farm air sample ('Real Env'; estimated amount of glyphosate: 8.66 μg/ml) and reagent grade glyphosate (Sigma–Aldrich, St. Louis, MO) (100 ng, 1 μg or 100 μg) were delivered (in 30μl) to the nose of anesthetized mice which were witnessed to aspirate the solution. Treatments were administered either: daily for 7 days or 3 times a week for 3 weeks. Same exposure schedule was followed for OVA alone (100 μg) and for OVA (100 μg) plus different dose of farm air sample and glyphosate. Mice were sacrificed 24h after final airway treatment with sodium pentobarbital.

### 2.7. Histological analysis of lung

Formalin–fixed paraffin embedded lung sections (5 μm thick) were prepared for H&E and chloroacetate esterase (CAE) staining. The entire histological slide from each mouse was examined in blinded fashion and given a global categorical severity score based on infiltration of cells into parenchymal, peribronchial, and perivascular regions of lungs.

### 2.8. Immunohistochemical staining

To analyze IL–33 and TSLP expression in the lungs section, the following antibodies were used for immunostaining: mouse IL–33 (0.2 mg/ml, AF3626, R&D Systems, Minneapolis, MN); mouse TSLP biotinylated (0.2 mg/ml, BAF555, R&D Systems) and respective isotype controls (R&D Systems). IL–33 and TSLP antibody–antigen complex were detected using Cy3 donkey anti–goat IgG (1:10000) (Invitrogen/ Molecular probes, Grand Island, NY). Slides were counterstained with DAPI (Vector Labs, Burlingame, CA). Images were obtained using a Nikon A1R si microscope.

### 2.9. Isolation of lung inflammatory cells

Lungs were perfused with PBS, removed, manually minced into 1–2 mm fragments and then placed in Hank's Balanced Salt Solution (Sigma–Aldrich) containing Liberase TL (50μg/ml; Roche Diagnostics, Indianapolis, IN) and DNase I (0.5mg/ml; Sigma–Aldrich). Tissue was digested at 37°C in a $CO_2$ incubator for 30 min. The tissue suspension was then passed through a 40 μm cell strainer. ACK lysis buffer (Invitrogen) was used to clear red blood cells.

### 2.10. Flow cytometric analysis

Single cell suspensions from lungs ($10^6$ per ml) were blocked with anti–mouse CD16/CD32 antibodies before cell–surface staining. Cells were stained with fluorescently– labeled

antibodies against SiglecF, Ly–6G/C (Gr–1), in different combinations according to the experiment. Analysis was performed using a FACSCanto II cytometer and FACSDIVA software (BD Biosciences). We defined eosinophils as being SiglecF$^{+}$Gr–1$^{+}$ and neutrophils as SiglecF$^{-}$ Gr–1$^{+}$.

### *2.11.* Cytokine measurement

IL–4, IL–10, IL–13, and IFN–γ production were measured by the *in vivo* cytokine capture assay (IVCCA) (Finkelman et al., 1999). Briefly, biotinylated cytokine–specific mAbs were injected via tail vein immediately before the last airway treatment, and blood was collected 24h later; sera or plasma were analyzed with microtiter plates wells coated with corresponding anti– cytokine mAbs. Cytokine levels were also assessed in bronchoalveolar lavage fluid (BALF) that was obtained 24h after the last airway treatment. A kit for measuring *in vivo* IL–4 production by IVCCA, R46A2 and XMG1.2 anti–IFN–γ mAbs was purchased from Becton–Dickinson (Franklin Lakes, NJ); eBio1316H and eBio13A anti–IL–13 mAbs, JES5–2A5 and JES5–16E3 anti–IL–10 mAbs, ELISA Ready–SET–Go analysis kits for measurement IL–33 and IL–5 were purchased from eBioscience. Assays were performed according to the kit's manufacturer protocols.

### *2.12.* Statistical analyses

Data were analyzed with Sigma Plot 12.0 (Systat Software, Inc., San Jose, CA). Statistically significant differences in means were determined by one–way ANOVA followed by Bonferroni multiple comparison tests. Kruskal–Wallis tests were conducted if the data did not have a normal distribution. All the data are presented as means ±SD for each group. Probability values of $<0.05$ were considered significant.

## 3. Results

### *3.1.* Exposure of air samples collected during glyphosate spray on farms stimulates airway inflammation

Wild type C57BL/6 (WT) and TLR4–/– mice were intranasally exposed to 'Real Env' samples (PBS suspended farm air samples) daily for 7days. 'Real Env' exposure was found to substantially increase the cell count in both the lungs and BAL fluid of WT and TLR4–/– mice. Additionally, the increase in pulmonary infiltrate in lungs was found to be higher in TLR4–/– than in WT mice (Fig. 1A and B). Similarly, we also observed an increase in eosinophil and neutrophil levels in 'Real Env' treated mice (Fig. 1C-F). This inflammation was also confirmed by histological examinations (Fig. 1G) and elevated IgG1 and IgG2a levels (Supplementary Fig. S1C and D).

Additional experiments were conducted using reagent grade glyphosate of different doses. Administration of reagent grade glyphosate to the airway of mice produced substantial pulmonary inflammation whether the daily dose given was 100ng,1 μg or 100 μg for 7days. In the BALF and lung digests, we found a significant increase in the total cell count when treated with glyphosate at 1 μg or 100 μg (Fig. 2A and D). Eosinophils (Fig. 2B and C), neutrophils, (Fig. 2E and F), and IgG1 and IgG2a levels (Supplementary Fig. S1A and B) were also increased in glyphosate–treated mice compared to controls. However, we did not

find any significant changes in the total cell count, eosinophils and neutrophils, IgG1 and IgG2a at glyphosate dose of 100 ng. Inflammation was confirmed by histological examination (Fig. 2M). Mice treated with both reagent grade glyphosate and OVA demonstrated significantly higher cell count (Fig. 2G and J), eosinophils (Fig. 2H and K), neutrophils (Fig. 2I and L), IgG1, and IgG2a (Supplementary Fig. S1A and B) compared to PBS treated mice.

Because pulmonary mastocytosis is typically observed in protein–allergen–induced experimental asthma, we assessed the pulmonary mast cell burden in our mice. We did not observe a significant increase in mast cell number in lungs treated with the substances isolated from the air on active farms ('Real Env') and reagent grade glyphosate (Fig. 3A and C; Supplementary Fig. S2). However, we did find the MCPT–1 levels to be substantially higher in both groups indicating increased mast cell degranulation in the treated mice (Fig. 3B and D).

### *3.2.* Glyphosate-rich farm air samples induced airway inflammation and higher production of IL–10, IL–13, IL–5, IFN–γ and IL–4 but glyphosate alone failed to produce IL–4

To evaluate the glyphosate–induced inflammation, we measured the systemic cytokine profile (Fig. 4A-E) of 'Real Env' and glyphosate exposed mice using IVCCA (Finkelman et al., 1999). We found significantly higher levels of IL–5, IL–10, IL–13, and IL–4 upon treatment with 'Real Env' alone in WT and TLR4–/– mice (Fig. 4A-D) approaching the levels induced by treating with OVA alone. The production of IL–5, IL–13 and IL–10 following 'Real Env' exposures was higher in TLR4–/– than in WT mice. We did not find any significant difference in IL–4 production between TLR4–/– and WT mice (Fig. 4D). We then tested production of these cytokines in mice given two different doses of glyphosate and found significantly higher levels of IL–5, IL–10, IL–13 and IFN–γ (Fig. 4F) that approached those levels induced by treating with OVA alone. Notably, there was no additional or synergistic effect when OVA was co–administered with glyphosate (Fig. 4G). Another interesting finding is that glyphosate alone was unable to induce significant levels of IL–4 while airway treatment with glyphosate with OVA did so.

### *3.3.* IL–33 and TSLP in lungs are increased upon exposure to glyphosate–rich air samples as well as reagent grade glyphosate alone

As the cytokine profile of mice treated with 'Real Env' and glyphosate approximated those treated with OVA, we looked at mediators known to promote type 2 pathology. IL–33 and TSLP appeared to be logical choices because of their well-recognized effector functions, and due to their source—the respiratory epithelium cells which would be the first cells to encounter inhaled glyphosate. We measured the IL–33 and TSLP content of BALF directly and found an abundance of both cytokines in 'Real Env'–treated WT and TLR4–/–mice (Fig. 5A and B). IL–33 production was observed to be significantly higher in TLR4–/– mice compared to WT mice. We also observed an abundance of both cytokines in glyphosate–treated mice (Fig. 5C and D). This finding was confirmed by immunohistochemical staining of IL–33 and TSLP in lung sections of glyphosate–treated mice (Fig. 5E) and 'Real Env'–treated WT and TLR4–/–mice (Supplementary Fig. S3A and B) which demonstrated

substantial production of both cytokines, which was limited to the respiratory epithelium after glyphosate exposure.

### *3.4.* Glyphosate–induced pulmonary inflammation is attenuated in IL–13 –/– mice

Glyphosate as a small molecule may not be efficiently presented to conventional T cells by antigen–presenting cells (Itano and Jenkins, 2003). The involvement of innate pathways upon glyphosate exposure, as we hypothesized, was supported by the absence of an increased production of IL–4. This absence would have been expected if type 2 innate lymphoid cells (ILC2s) were the primary source of the IL–5 and IL–13 detected.

IL–33 and TSLP have been well described to induce ILC2s, which in turn causes lung pathology particularly via IL–13–dependent mechanism. To test this hypothesis, we exposed IL– 13 deficient mice to glyphosate for 7 and 21 days and assessed lung inflammation. While there was no change in IL–4 levels, we found that the inability to produce IL–13 prevented the rise in IL–5 production, but not the rise in IL–10 production, at both time points during glyphosate treatment. Deficiency in IL–13 also prevented a significant rise in IL–33 and TSLP levels at the early time point but not the latter one (Fig. 6A-D). Lack of IL–13 production was also associated with significantly less ($P < 0.05$) severe cellular infiltration noted on histology (Fig. 6E). Despite significant inflammation, we did not find airway hyperresponsiveness in glyphosate–treated wild type and IL–13–/– mice (Supplementary Fig. S4A and B).

## 4. Discussion

In the present study, we explored the ability and the mechanisms of airway asthma-like pathology caused by glyphosate-rich environmental samples collected on farms as well as glyphosate in a pure form. The data presented in this work for the first time demonstrate substantial airway inflammation upon exposure to farm air samples containing glyphosate as well as exposure to glyphosate alone. Notably, farm air samples and low dose of glyphosate provoked a mixed response with elevated pulmonary eosinophils and neutrophils.

Increasing the dose of glyphosate 100–fold up to 100 μg did not substantially change the degree or character of inflammation; however, a longer exposure to glyphosate did significantly worsen histological pathology. No change of inflammatory response at higher dose is explainable because of the difference between inflammatory immune responses and toxic reactions. A toxic effect is unswervingly the result of the toxic chemical acting on cells. On the other hand, inflammatory responses are the result of a chemical stimulating the body to liberate natural chemicals (e.g., cytokines) which are in turn directly responsible for the effects observed. Thus, in an inflammatory reaction, the chemical can act simply as a trigger, but not as the bullet. In addition, the higher doses of glyphosate could exert some toxic effect on epithelial cells making them unresponsive to elicit allergic inflammatory responses.

In our study we observed minor or none exacerbation of immune response upon co-exposure of glyphosate and common allergen ovalbumin as well as upon glyphosate treatment alone. This was surprising due to a significant increase of IL-33 and TSLP expression upon

glyphosate treatment. Our results could be explained by a possibility that upon exposure to ovalbumin there is not enough damage of airway epithelial cells to release sufficient amount of IL-33 and TSLP. Other airborne allergens such as house dust mite that share the proteinase activity could be much more efficient in releasing of large amount of danger signals that could result in asthma exacerbation. There is also an unanswered question if glyphosate can exacerbate established asthma. The role of glyphosate is controversial due to the toxic properties of the herbicide that may result in immunosuppressive effects versus its ability to induce significant innate cytokine response. More studies are needed to elucidate these relevant questions.

Interestingly, there was no diminishment in the immune responses in TLR4−/−mice upon exposure to farm air samples. However, these mice demonstrated a tendency to generate more IL–13, IL–4 and IL–10. The possible reason could be the decreased toleration ability in TLR4−/− mice as has been shown in another allergy related study (Pochard et al., 2010). Taking into consideration the TLR4 polymorphism in the human population (Kerkhof et al., 2010; Kumar et al., 2012; Belforte et al., 2013; Vawda et al., 2014), it may be a significant risk factor for allergic symptoms in farmers.

Several previous studies have suggested cytotoxic and/or genotoxic effects of glyphosate on human cells (Richard et al., 2005; Monroy et al., 2005; Benachour et al., 2007; Benachour and Séralini, 2009). Reports also showed DNA damage and genotoxicity among individuals two months after their exposure to aerial spraying of glyphosate (Paz-y-Mĩno et al., 2007; Koller et al., 2014). However, the immunological consequences of glyphosate upon the airway have been rarely investigated experimentally. The findings from this study will help both researchers and clinicians to better understand the mechanisms of occupational asthma caused by one of the most extensively applied herbicides.

Since the asthma phenotype associated with occupational use of glyphosate is reported to be "atopic", we assessed the cytokine profile produced in the murine lung and found canonical type 2 cytokines IL–5 and IL–13 were increased, but not IL–4 – both at early and later time points. We observed T-cell mediated antigen-specific antibody response in mostly IL-4 independent manner. Production of type 2 cytokines such as IL–5 and IL–13 in IL–4–independent manner has also been found to be associated with allergic disorders in a study conducted by Kurowska–Stolarska et al. (2008). As we also found that the condition induced by glyphosate exposure lacks the robust goblet cell metaplasia (data not shown) and lacks substantial AHR, the clinical implication would be that the inflammation produced by glyphosate in humans may be relatively asymptomatic—but could enhance symptoms of wheeze and cough in the allergic human lung.

To understand the mechanism of pulmonary inflammation and type 2 cytokine responses that we saw in our study, we wanted to look at IL–33 and TSLP in this experimental approach which had not been done before. The reasons for suspecting that IL–33 might be important in glyphosate–mediated airway disease were: 1) IL–33 is known to induce TNF–α, IFN–γ and IL–13 upon antigen challenge followed by activation and recruitment of inflammatory cells in the airways (Brightling et al., 2002), 2) IL–33 induced expression of IL–13 leads to severe pathological changes in mucosal organs (Schmitz et al., 2005), 3) IL–

33 enhances the eosinophil activation *in vitro* (Suzukawa et al., 2008), and increase airway inflammation *in vivo* (Kondo et al., 2008), 4) IL–33 is involved in the induction of allergic inflammatory responses by promoting pulmonary eosinophilia, IL–5, IL–13, and IgE (Smith, 2010; Liew et al., 2010), and 5) IL–33 is crucial for the induction of type 2 inflammation through innate pathways and is associated with tissue damage (Oboki et al., 2010). We observed increased IL-33 expression in both BALFs and lung sections, suggesting IL-33 may play an important role in innate immune response as well as in airway inflammation accompanied by significant accumulation of eosinophils caused by glyphosate exposure.

The involvement of TSLP has been similarly implicated in the mechanisms of pulmonary inflammation (Soumelis et al., 2002; Miyata et al., 2008). Furthermore, TSLP has also been found to be associated with the development and exacerbation of airway inflammation in mice (Zhou et al., 2005; Harada et al., 2009). When we examined TSLP and IL–13 levels in IL–13– deficient mice, their rise upon glyphosate treatment was delayed as compared to wild type mice – the rise being present at 21 days but not at 7 days. The reason for this is unclear, but suggests that: 1) IL–33/TSLP–mediated induction of type 2 cytokines is not necessarily a unidirectional process—at least in the absence of a classically activated adaptive immune response, 2) IL–13 appears to provide some degree of positive feedback for the propensity to release IL–33/TSLP from the epithelium, and 3) this deficit in IL–33/ TSLP secretion associated with IL–13 deficiency can be overcome by further stimulation of glyphosate or inflammatory factors derived from the inflammatory milieu.

A second unexpected finding that recapitulates the central role of IL–13 in type 2 pathophysiology is that rise in IL–5 levels was not observed in mice deficient in IL–13 at both the early and late time points of glyphosate treatment. It is unclear how to interpret this finding, but this rise would presumably involve the overall decreased state of inflammation.

We observed a significant elevation of IL–10 production upon exposure to glyphosate. This is not an unexpected finding considering a significant ongoing inflammatory response. The elevated levels of IL–10 and possibly increased kinetics of its production were not sufficient to control the inflammation.

Collectively, our results showed that mice continuously exposed to glyphosate developed elevated levels of eosinophils, neutrophils, and asthma-related cytokines (IL-5, IL-10, IL-13, IL-33, TSLP) compared to control groups. Exposure to glyphosate results in airway barrier damage. This damage induces IL-33 and TSLP innate cytokines release that may have a sensitization role; co-exposure with an allergen leads to a profound inflammatory and antigen-specific innate and adaptive immune response, including release of IL-13, IL-5, IL-10, and antigen-specific antibodies. All these events could be a possible explanation of how glyphosate contributes to an induction and/or exacerbation of asthma-like airway pathology. Further studies are needed to explore the mechanistic role of glyphosate in triggering allergic inflammation eventually leading to asthmatic symptoms in agricultural workers and other populations exposed to higher levels of glyphosate, which is now widely used as a conventional herbicide around the world.

## 5. Conclusions

Our results demonstrate the capacity of glyphosate-rich air samples from farms as well as pure glyphosate to induce type 2 airway inflammation, over both short and longer time courses. Furthermore, glyphosate induced inflammation was found to be associated with induction of IL-33 and TSLP. This work also highlights the production of IL–13 as well as modulation of innate immune system by glyphosate, which may play an important role in exacerbation of airway inflammation by this low molecular weight chemical.

## Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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## Highlights

- Glyphosate-rich air samples induce antigen-independent airway inflammation.
- Glyphosate causes high expression of IL-33 and TSLP during the airway inflammation.
- Glyphosate exposure in airways produces canonical Th2 cytokines.
- Glyphosate-associated airway inflammation is partially dependent on IL-13.
- IL-13, TSLP, IL-33 can be potential targets to control glyphosate-induced inflammation.

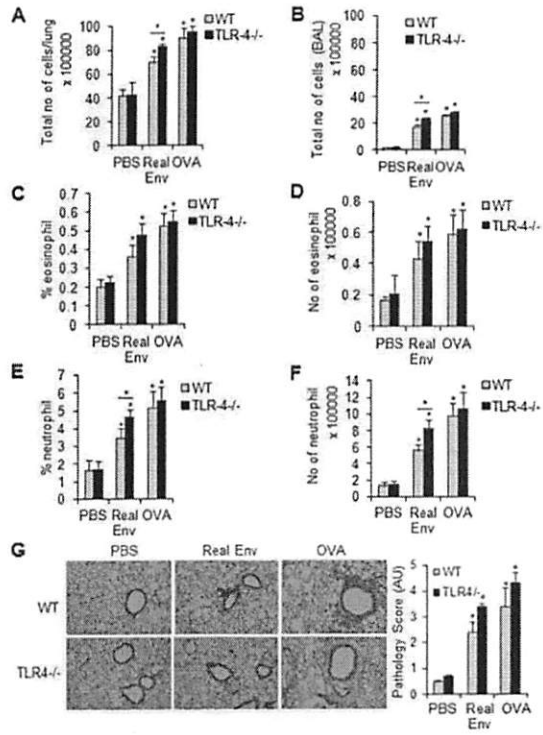


**Fig. 1.**

Increase in total number of cells, eosinophils, and neutrophils in lung and BAL fluids upon airway exposure to farm air samples ('Real Env') and OVA for seven consecutive days (mean ± SD; n = 8). Increase in total number of cells in (A) lung and (B) BAL fluids. Increase in percentage (C) and total number (D) of eosinophils and neutrophils (E, F) per lung upon exposure to farm air samples ('Real Env'). (G) Representative lung sections (H&E staining) and its pathology score from mice treated with PBS, farm air samples ('Real Env') and OVA intranasally for seven consecutive days (mean ± SD; n = 8); magnification 200X.

* indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4−/− mice group.

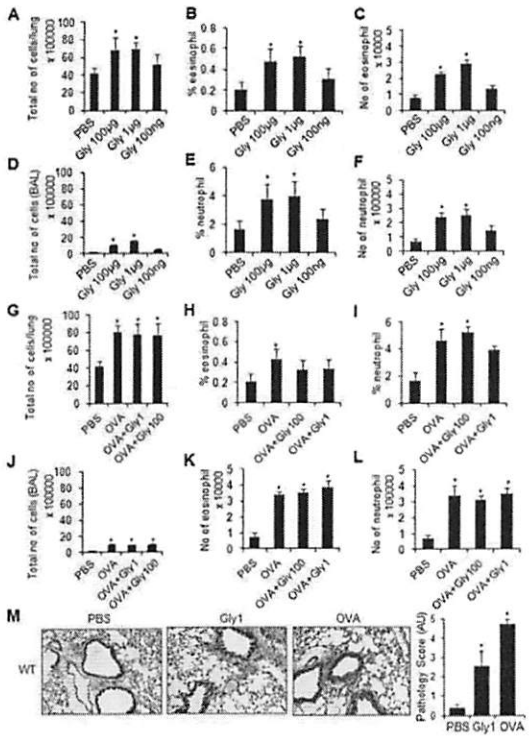


**Fig. 2.**

Increase in total number of cells,eosinophils, and neutrophils in lung and BAL fluids of WT mice upon airway exposure to glyphosate and combinations of glyphosate and OVA for seven consecutive days (mean ± SD; n = 8). Increase in total number of cells in (A) lungs and (D) BAL fluids upon exposure to different doses of glyphosate (100 ng, 1, or 100 µg). Increase in percentage (B) and total number (C) of eosinophils and neutrophils (E, F) per lung upon exposure to two doses of glyphosate. Increase in total number of cells in (G) lungs and (J) BAL fluids upon exposure to combination of glyphosate (1 or 100 µg) with OVA (100 µg). Increase in percentage and total number of (H, K) eosinophils and (I, L) neutrophils per lung upon exposure to OVA and combination of glyphosate, respectively. (M) Representative lung sections (H&E staining) and its pathology score from WT mice treated with PBS, glyphosate (1µg) and OVA (100 µg) intranasally for seven consecutive days (mean ± SD; n = 8); magnification 200X.

* indicates statistically significant differences ($p < 0.05$) with respect to PBS and treated WT mice group.

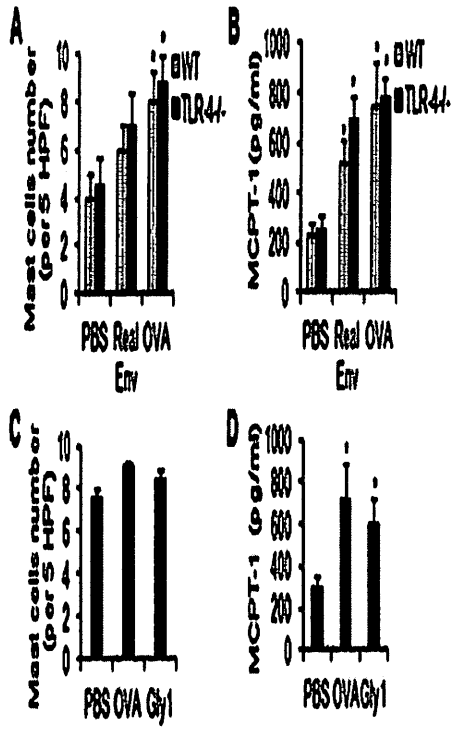


**Fig. 3.**

Farm air samples containing glyphosate as well as pure glyphosate alone induce increased mast cell degranulation but no increase in lung mast cell numbers upon airway exposure. (A) Mast cells number in CAE stained lung section and (B) serum MCPT–1 concentration in blood 4h after last exposure of PBS, farm air samples ('Real Env'), and ovalbumin (OVA). (C) Mast cells number in CAE stained lung section and (D) serum MCPT–1 concentration from mice treated with PBS, ovalbumin and 1 μg of glyphosate deliverd to intranasally for seven consecutive days (mean ± SD; n = 8).

* indicates statistically significant differences ($p < 0.05$) with respect to PBS and treated mice group.

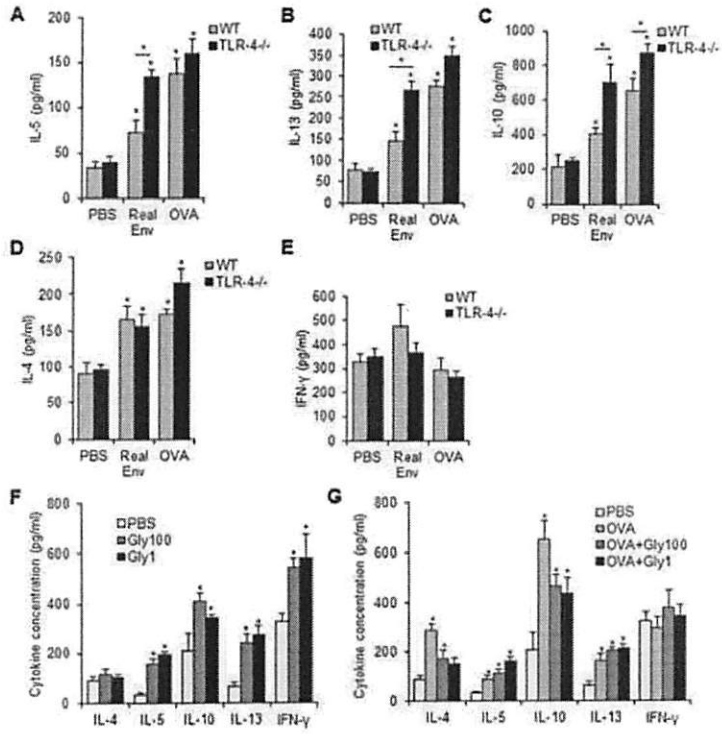


**Fig. 4.**

(A–E) Higher production of IL–5, IL–13, IL–10, IL–4 and no change in the IFN–γ levels upon exposure to farm air samples in WT and TLR4−/− mice. (F) The increased level of IL–5, IL–10, IL–13, IFN–γ and no change in the IL–4 levels upon glyphosate (1 or 100 μg) exposure to WT mice. (G) The increased level of IL–4, IL–5, IL–10, IL–13, and no change in IFN–γ levels upon combination of glyphosate (1 or 100 μg) and ovalbumin (100 μg) exposure to WT mice (mean ± SD; n = 8). Levels of cytokines were evaluated by IVCCA in serum of mice upon 7 consecutive days of intranasal treatment with farm air samples ('Real Env') and glyphosate. Blood samples were collected 24h after the last exposure. IL–5 was measured in the BAL fluids.

* indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4−/− mice group.

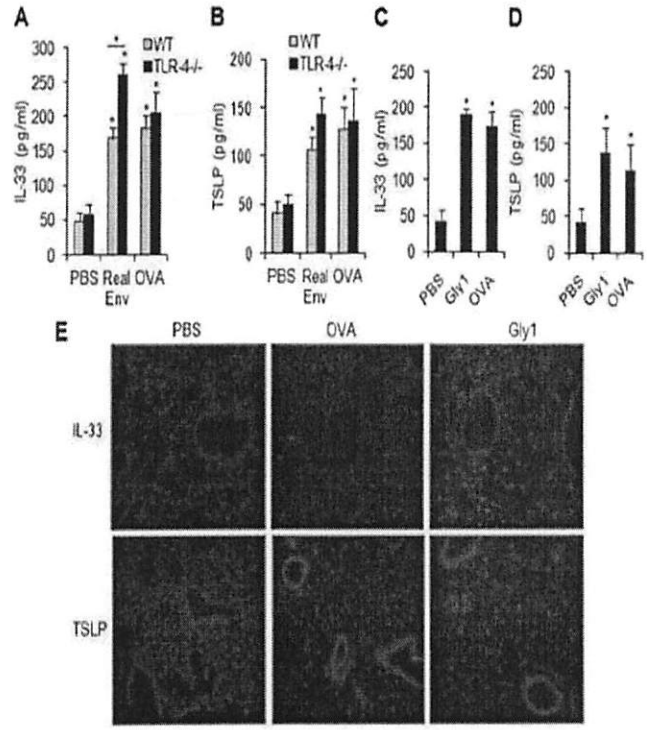


**Fig. 5.**

IL–33 and TSLP productions increased in the lung upon exposure to farm air samples and glyphosate. (A, B) ELISA based measurement of IL–33 and TSLP in BAL fluids of PBS, farm air samples and ovalbumin (100 μg) treated WT and TLR4 −/− mice, respectively (mean ± SD; n = 8). (C, D) ELISA based measurement of IL–33 and TSLP in BAL fluids of PBS, OVA and pure glyphosate (1 μg) treated WT mice, respectively (mean ± SD; n = 8). (E) Immunofluorescence staining of IL–33 and TSLP in the lung sections of the glyphosate treated WT mice, magnification 200X.

* indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control and in between WT and TLR4−/− mice group.

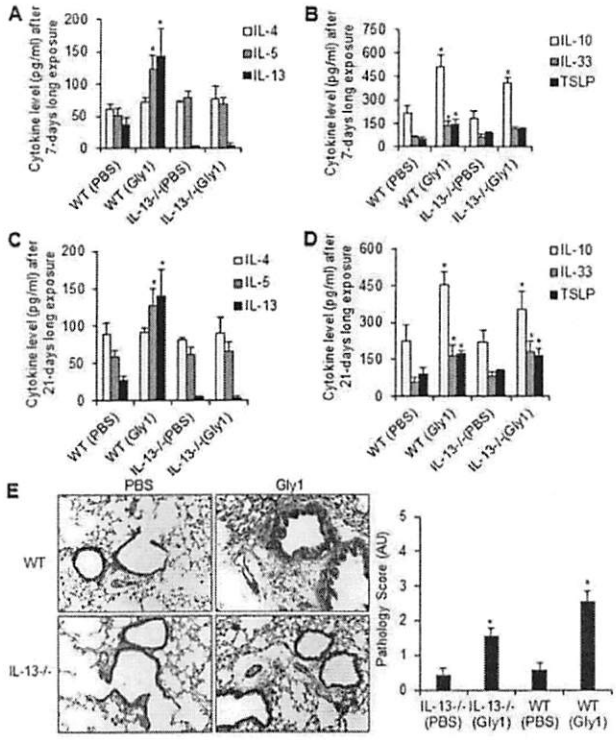


**Fig. 6.**

IL–13–deficient mice demonstrated diminished inflammatory response upon glyphosate exposure. (A, C) Diminished production of IL–5 but no change in IL–4 level, and (B, D) diminished production of TSLP, IL–33, IL–10 levels, between IL–13–deficient mice and WT mice upon glyphosate exposure (1μg) for 7 or 21 days, respectively (mean ± SD; n = 8). (E) Representative lung sections (H&E staining) from mice treated with PBS and glyphosate (1 μg) intranasally three times a week for 21 days; magnification 200X (left panel). Arbitrary scores were based on inflammatory cells infiltration in lungs parenchyma, peribronchial, and perivascular regions. Analysis was performed in a double blinded manner (right panel).

* indicates statistically significant differences ($p < 0.05$) with respect to PBS treated control group.

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# Pesticides are Associated with Allergic and Non-Allergic Wheeze among Male Farmers

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BACKGROUND: Growing evidence suggests that pesticide use may contribute to respiratory symptoms.

OBJECTIVE: We evaluated the association of currently used pesticides with allergic and non-allergic wheeze among male farmers.

METHODS: Using the 2005–2010 interview data of the Agricultural Health Study, a prospective study of farmers in North Carolina and Iowa, we evaluated the association between allergic and non-allergic wheeze and self-reported use of 78 specific pesticides, reported by ≥ 1% of the 22,134 men interviewed. We used polytomous regression models adjusted for age, BMI, state, smoking, and current asthma, as well as for days applying pesticides and days driving diesel tractors. We defined allergic wheeze as reporting both wheeze and doctor-diagnosed hay fever ($n$ = 1,310, 6%) and non-allergic wheeze as reporting wheeze but not hay fever ($n$ = 3,939, 18%); men without wheeze were the referent.

RESULTS: In models evaluating current use of specific pesticides, 19 pesticides were significantly associated ($p < 0.05$) with allergic wheeze (18 positive, 1 negative) and 21 pesticides with non-allergic wheeze (19 positive, 2 negative); 11 pesticides were associated with both. Seven pesticides (herbicides: 2,4-D and simazine; insecticides: carbaryl, dimethoate, disulfoton, and zeta-cypermethrin; and fungicide pyraclostrobin) had significantly different associations for allergic and non-allergic wheeze. In exposure–response models with up to five exposure categories, we saw evidence of an exposure–response relationship for several pesticides including the commonly used herbicides 2,4-D and glyphosate, the insecticides permethrin and carbaryl, and the rodenticide warfarin.

CONCLUSIONS: These results for farmers implicate several pesticides that are commonly used in agricultural and residential settings with adverse respiratory effects.

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## Introduction

Worldwide, more than 5 billion pounds of pesticide active ingredients are used annually (U.S. EPA 2011a). Pesticides represent a diverse group of chemical and physical agents that have varying toxicity for both plants and animals. Pesticide exposure is common in both agricultural and residential settings; approximately 15% of the insecticides and 8% of herbicides used in the United States are for residential use. Many of the same chemicals are used in both agricultural and residential settings (U.S. EPA 2011a).

Although little is known about the potential respiratory impact of most currently used pesticides, evidence is growing that pesticide exposures contribute to respiratory symptoms and asthma. Case reports suggest that organophosphate insecticides (Bryant 1985; Weiner 1961), 2,4-D (Forbes et al. 1966), and pyrethroids (Lessenger 1992; Newton and Breslin 1983; Wagner 2000) are associated with asthma or asthmatic symptoms. Epidemiologic studies of farmers, farmworkers and commercial pesticide applicators have linked specific pesticides, including paraquat, chlorpyrifos and other organophosphates, and pyrethroids to asthma and wheeze (Castro-Gutiérrez et al. 1997; Fieten et al. 2009; Hoppin et al. 2002a, 2006, 2008, 2009; Liu et al. 2012; Ohayo-Mitoko et al. 2000; Raanan et al. 2015). Biologic mechanisms have been evaluated in animal studies for specific chemicals, including paraquat, carbaryl, and some organophosphate insecticides (Cho et al. 2008; Dong et al. 1998; Fryer et al. 2004). Large epidemiologic studies provide the opportunity to focus on specific chemicals rather than general categories of pesticide exposure such as insecticides.

To evaluate whether currently used pesticides are associated with respiratory symptoms, we used recent data from the Agricultural Health Study (AHS), a prospective study of licensed pesticide applicators and their spouses in Iowa (IA) and North Carolina (NC) (Hoppin et al. 2014). In the 2005–2010 interview, participants provided information on their current pesticide use as well as recent respiratory symptoms. Building on our earlier work (Hoppin et al. 2002a, 2006, 2008, 2009) and animal data that suggest differential effects for allergen-sensitized animals (Proskocil et al. 2008), we evaluated associations of current pesticide use with both allergic and non-allergic wheeze in male pesticide applicators. This paper includes updated analyses for 27 of the 40 pesticides previously evaluated for wheeze (Hoppin et al. 2002a, 2006) as well as initial analyses for 51 additional pesticides; chemicals with infrequent or no use during this period were not evaluated.

## Materials and Methods

### Population

We assessed pesticide exposures and wheeze among male participants in the AHS who completed the 2005–2010 follow-up interview

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We used the following AHS (Agricultural Health Study) data releases for this analysis: Phase 1: P1REL201209.00, Phase 2: P2REL201209.00, Phase 3: P3REL201209.00, Demographic/Mortality: AHSREL201209.00. We thank M. Dunn and S. Legum at Westat (Rockville, MD) for their study and data management.

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(Hoppin et al. 2014). Participants completed a computer-aided telephone interview that collected information on current farming activities, pesticide use, medical conditions, and other demographic factors. The response rate was 51% for farmers, with the majority of nonresponse associated with our inability to contact them by phone (Hoppin et al. 2014). Questionnaires are available at http://aghealth.nih.gov/collaboration/questionnaires.html. All individuals with data on wheeze and hay fever as well as all model covariates were included. We restricted the sample to men as both the exposure profile and the risk factors differed by gender in the AHS. The AHS has been reviewed and approved by the Institutional Review Boards of the National Institues of Health (NIH) and its contractors. All participants in the AHS provided informed consent when they enrolled in the study.

### Outcome

The outcome in our analyses had three levels: no wheeze (controls), allergic wheeze, and non-allergic wheeze. This three-level outcome allowed us to assess differential response to pesticides based on allergic status. We defined a participant as having wheeze if he reported having at least one episode of wheeze or whistling in his chest in the past year. We assigned allergic status based on a history of doctor-diagnosed hay fever. Our previous work in this cohort (Hoppin et al. 2002a, 2006, 2008, 2009) used both eczema and hay fever as markers of allergy, but the current questionnaire did not collect information on eczema (and not all participants responded to the earlier questionnaire including eczema). In earlier work, hay fever alone accounted for 70% of those classified with allergic wheeze, thus our definitions are similar in this adult population. We considered individuals with both wheeze and allergy to have allergic wheeze and those with wheeze alone to have non-allergic wheeze. Controls were individuals without wheeze, irrespective of their allergy status because we were interested in allergy as a modifier of wheeze, not as an outcome.

### Exposure Assessment

Participants provided information on their current pesticide use through open-ended questions that asked about pesticide use since their last interview. Individuals provided verbatim names (usually trade names) of the pesticides that they used on crops, animals, and for non-crop purposes. These verbatim names were then linked to pesticide active ingredients' names using the U.S. Environmental Protection Agency (EPA) Pesticide Classification Code (PC Codes; see https://www.epa.gov/ingredients-used-pesticide-products/how-search-information-about-pesticide-ingredients-and-labels) to generate a list of 515 pesticides used by participants since the last interview (Hoppin et al. 2012). Participants also provided information on the frequency (days/year) and duration (years) of use. For this analysis, we limited our evaluation to the 78 pesticides used by at least 1% of those completing the questionnaire ($n$ = 22,134). Those 78 pesticides included 45 herbicides and plant growth regulators, 25 insecticides, 6 fungicides, 1 fumigant, and 1 rodenticide. A few pesticides were most often used in combination as a result of being part of the same product; therefore, there was a high degree of correlation between these chemicals, and little ability to discriminate one pesticide from the other. Notably, two pairs of herbicides had this characteristic: *a*) fenoxaprop-p-ethyl and fluazifop-butyl and *b*) clopyralid and flumetsulam. For these pairs of herbicides, we report only one set of estimates with current exposure defined as current use of either and days applied equal to the greater of the two chemicals, if they differed.

We used the current pesticide use information, along with previously reported pesticide use from the earlier interviews to create three-level pesticide use variables: current use (since the last interview), past use (not used in current cycle), and never use. We included past use as a separate category so that never users of that chemical would be the referent category, because farmers who wheezed in response to use of a specific pesticide may have stopped using that pesticide in subsequent years.

### Statistical Methods

We used polytomous regression models adjusted for current age (categories: < 50, 50–59, 60–69, 70–97 years), body mass index (BMI, < 25, 25–30, > 30), smoking status (current, past, never), current asthma (yes/no), and state (NC, IA) as well as for two current farming-related variables associated both with wheeze and pesticide use: days/year mixed or applied any pesticides (0, 1–10, 11–365 days, based on the median days of annual pesticide application) and days/year driving diesel tractors (0, < 31, 31–90, ≥ 90 days, based on questionnaire categories) to evaluate the association between pesticide exposure and allergic and non-allergic wheeze. We defined current asthma based on self-reported doctor's diagnosis of asthma and a positive response to "Do you still have asthma?" We have used similar models in our previous analyses in this cohort (Hoppin et al. 2002a, 2006). The polytomous regression models allowed us to formally test the differences between the odds ratios (ORs) for allergic and non-allergic wheeze using a Wald test for the contrast between the two log OR parameters; a $p$-value for difference was the result of this contrast test. To assess whether correlation among pesticides could explain our findings, we assessed pairwise correlations between chemical-specific current use variables for chemicals that were significantly associated with wheeze. If the correlation exceeded 0.3, we included both chemicals in the model. All analyses were done using SAS software (version 9.3; SAS Institute Inc., Cary, NC). We used PROC LOGISTIC with the GLOGIT option for multi-level response variables and the CONTRAST statement to evaluate differences between levels.

We estimated OR for allergic and non-allergic wheeze in separate models for each chemical. Current pesticide use was parameterized in two ways: *a*) any current use and *b*) frequency of current use in categories. To create categories of use for exposure–response modeling, we divided the distribution of users into tertiles based on frequency of use; then we further subdivided the top third either in half or thirds depending on the number of users of that chemical. At a minimum, there were 10 exposed cases in each category. This modeling strategy allowed us to better evaluate the high end of the exposure distribution in this population where many individuals used chemicals for only 1 or 2 days a year.

Wheeze is the cardinal symptom of asthma and as a result most asthmatics report wheeze, though many more people report wheeze than have asthma. To assess whether inclusion of asthmatics in our models influenced our findings, we reran our models excluding those with current asthma to evaluate whether asthmatics were driving the effect estimates.

## Results

Of the 22,134 male pesticide applicators who completed the 2005–2010 interview, 1,310 (6%) had both wheeze and allergy, "allergic wheeze," whereas 3,939 (18%) reported only wheeze "non-allergic wheeze" (Table 1). Individuals from Iowa were more likely to report non-allergic wheeze, while those in NC were more likely to report allergic wheeze. Younger farmers (< 50 years) were more likely to wheeze than older farmers. Current smokers, individuals with higher BMIs, and current asthmatics were more likely to wheeze. Although asthma was more common among those with wheeze, only 27% of those with allergic wheeze and 8% of those with non-allergic asthma reported current asthma. Farmers who applied pesticides more often and those who drove diesel tractors were more likely to report wheeze.

We evaluated current use of 78 pesticides in relation to allergic and non-allergic wheeze.

Overall, 29 pesticides had some association with at least one type of wheeze, 19 were significantly associated with allergic wheeze, 21 were associated with non-allergic wheeze, and 11 pesticides were significantly associated with both. Seven had ORs for allergic and non-allergic wheeze that differed statistically ($p < 0.05$) from each other, including the commonly used herbicide 2,4-D, which had an elevated OR for allergic wheeze [OR = 1.46, 95% confidence interval (CI): 1.19, 1.79], but not for non-allergic wheeze (OR = 1.12, 95% CI: 0.99, 1.26). For ease of presentation and discussion, we have organized the results by functional group (e.g., herbicides, insecticides) in Tables 2–4. In Figure 1, we provide an overview of all the statistically significant findings from Tables 2–4. In Table 5, we present exposure–response models for 10 commonly used pesticides; all exposure–response models appear in Excel File S1.

### Herbicides and Plant Growth Regulators

Of the 43 herbicides and two plant growth regulators, 18 were associated with at least one wheeze outcome (Table 2). Only one was inversely associated with wheeze, and it was inversely associated with both allergic and non-allergic wheeze (clomazone). Fourteen herbicides were positively associated with non-allergic wheeze and 10 with allergic wheeze. Three herbicides were associated only with allergic wheeze, while seven herbicides were associated only with non-allergic wheeze. Two herbicides (2,4-D and simazine) had statistically significant contrasts ($p < 0.05$) and were positively associated only with allergic wheeze.

Wheeze prevalence differed by herbicide chemical group. All three acetic acid herbicides (clopyralid/flumetsulam, picloram, and dicamba) were significantly associated with non-allergic wheeze and both picloram and dicamba were associated with allergic wheeze as well. Two of the three chloracetanilide herbicides (acetochlor and metolachlor) were significantly associated with non-allergic wheeze, and the third alachlor had an elevated but non-significant OR for non-allergic wheeze (1.25, 95% CI: 0.99, 1.58); there was no evidence of an association with allergic wheeze for this class of herbicides. Of the two dinitroaniline herbicides, only trifluralin was associated with non-allergic and allergic wheeze. Pendimethalin was not associated with wheeze. Of the three imidazolinone herbicides, only imazaquin was associated with wheeze and only significantly with non-allergic wheeze. Glyphosate, the most commonly used herbicide, was significantly associated with both types of wheeze, while the related but less commonly used glufosinate ammonium was not associated with either wheeze outcome. Of the three phenoxy herbicides, 2,4-D was significantly associated with allergic wheeze, while the fluazifop-butyl/fenoxaprop-p-ethyl was associated with non-allergic wheeze. The two triazines (atrazine, simazine) were significantly associated with allergic wheeze, while the related metribuzin (a triazinone) also had an elevated but not statistically significant association with allergic wheeze. Atrazine, but not simazine or metribuzin, was also associated with non-allergic wheeze. Use of petroleum distillates had the highest odds of wheeze for both non-allergic (OR = 1.61, 95% CI: 1.15, 2.25) and allergic wheeze (OR = 2.45, 95% CI: 1.50, 4.03).

### Insecticides

Current use of nine of the 25 individual insecticides was positively associated with at least one type of wheeze, and one insecticide (disulfoton) was inversely associated with non-allergic wheeze (Table 3). Two of the pyrethroids (permethrin, pyrethrins) were significantly associated with both allergic and non-allergic wheeze, while a third, zeta-cypermethrin was strongly associated with allergic wheeze (OR = 2.02, 95% CI: 1.24, 3.30) and inversely, but not significantly so, with non-allergic wheeze (OR = 0.88, 95% CI: 0.60, 1.30; $p_{contrast} = 0.005$). Of the organophosphates, malathion was associated with both allergic and non-allergic wheeze, while chlorpyrifos and dimethoate were associated only with allergic wheeze. Carbaryl, a carbamate insecticide, was associated with allergic (OR = 1.70, 95% CI: 1.32, 2.19), but not non-allergic wheeze (OR = 1.03, 95% CI: 0.87, 1.22; $p_{contrast} = 0.001$). Fly spray use was associated with non-allergic wheeze.

### Fungicides, Fumigant, and Rodenticide

In addition to herbicides and insecticides, we evaluated six fungicides, one fumigant, and one rodenticide for association with wheeze (Table 4). Of these, only the rodenticide warfarin was significantly associated with allergic wheeze (OR = 1.55, 95% CI: 1.04, 2.30), none of the chemicals were associated with non-allergic wheeze. Additionally, while not statistically significant, the fungicide pyraclostrobin was positively associated with allergic wheeze (OR = 1.46, 95% CI: 0.99, 2.14) but not with non-allergic wheeze (OR = 0.94, 95% CI: 0.72, 1.23; $p_{contrast} = 0.045$).

### Correlated Pesticides

Because pesticides can be used in combination or on the same crop over the course of a growing season, we evaluated whether correlation among the pesticides were responsible for the large number of significant findings. Among the pesticides associated with at least one category of wheeze, there were 27 pairs of Spearman correlations for pesticides that exceeded 0.3. Twelve pesticides

**Table 1.** Demographic, medical, and selected farming characteristics by wheeze status among 22,134 male pesticide applicators in the Agricultural Health Study, 2005–2010.

| Characteristic | Controls $n = 16,885$ % | Allergic wheeze $n = 1,310$ % | Non-allergic wheeze $n = 3,939$ % |
|---|---|---|---|
| Age at last interview (years) | | | |
| < 50 | 21 | 23 | 25 |
| 50–59 | 30 | 33 | 30 |
| 60–69 | 26 | 23 | 24 |
| 70–97 | 24 | 22 | 20 |
| State | | | |
| Iowa | 67 | 59 | 70 |
| North Carolina | 33 | 41 | 30 |
| Smoking status | | | |
| Never | 54 | 50 | 49 |
| Past | 39 | 40 | 37 |
| Current | 7 | 11 | 13 |
| Current asthma | 2 | 27 | 8 |
| Hay fever diagnosis | 14 | 100 | 0 |
| BMI | | | |
| < 25 | 20 | 15 | 16 |
| 25–30 | 56 | 51 | 51 |
| > 30 | 24 | 35 | 33 |
| Days/year mixed applied pesticides | | | |
| None | 32 | 28 | 28 |
| 1–10 | 37 | 37 | 38 |
| 11–365 | 31 | 35 | 34 |
| Days/year drive diesel tractors | | | |
| None | 18 | 17 | 14 |
| < 31 | 19 | 21 | 18 |
| 31–90 | 27 | 26 | 28 |
| ≥ 91 | 36 | 36 | 39 |

(nine herbicides: 2,4-D; acetochlor, atrazine, dicamba, glyphosate, mesotrione, metolachlor, nicosulfuron, trifluralin; three insecticides: dimethoate, disulfoton, and malathion) contributed to these 27 pairs. When we included the correlated pairs of pesticides in the same model, we saw no strong evidence of confounding with only small changes in the ORs for the individual chemicals (data not shown).

### Exposure–Response Modeling

We constructed exposure–response models for all pesticides. Ten chemicals (seven herbicides, three insecticides) had sufficient numbers of frequent users to create five-level exposure variables where the top third was itself split into thirds. Nine chemicals had four categories of exposure; the remaining 59 were split at tertiles of current use (see Excel File S1). Table 5 presents exposure–response models for 10 of the 78 pesticides, which we chose based on the frequency of use and evidence of an association with the outcome. The herbicides 2,4-D and glyphosate both showed higher prevalence of allergic wheeze with increasing use. While both also showed an association with non-allergic wheeze, the association for 2,4-D was only present among those who used 2,4-D at least 16 days/year. Atrazine had similar exposure–response profiles for both allergic and non-allergic wheeze, with increasing ORs up to 14 days per year and with a lower OR in the highest exposure category. Among the insecticides, carbaryl showed the greatest difference between allergic and non-allergic wheeze, with any level of use associated with increased allergic wheeze, but not with increased non-allergic wheeze. The organophosphates malathion and chlorpyrifos both showed elevated odds of allergic wheeze, but there was no strong evidence of an exposure–response relationship. Use of permethrin was associated with allergic and non-allergic wheeze with the highest odds associated with the highest level of use (13–365 days). The rodenticide warfarin was associated with increased allergic wheeze with increasing use.

When we limited the analysis to those without current asthma, we observed essentially the same findings for both allergic and non-allergic wheeze. Seven pesticides (herbicides: 2,4-D; acetochlor; and simazine; insecticides: carbaryl, tebupirimfos, dimethoate, zeta-permethrin) had significantly different estimates for allergic and non-allergic wheeze, but the estimates were similar to those for the whole sample (data not shown).

## Discussion

Our study evaluated a more comprehensive list of currently used pesticides in relation to wheeze than has ever been evaluated for any

**Table 2.** Current use of herbicides and plant growth regulators and odds ratios for allergic and non-allergic wheeze among 22,134 male participants in the Agricultural Health Study, 2005–2010.

| | Controls ($n$ = 16,885) | Allergic wheeze ($n$ = 1,310) | | Non-allergic wheeze ($n$ = 3,939) | | |
|---|---|---|---|---|---|---|
| Chemical name and group | Current users % | Current users % | OR (95% CI) | Current users % | OR (95% CI) | $p$-Value contrast* |
| Acetic acid herbicide | | | | | | |
| Clopyralid/flumetsulam | 5 | 5 | 1.12 (0.86, 1.45) | 7 | 1.33 (1.16, 1.54) | |
| Dicamba | 12 | 13 | 1.28 (1.04, 1.58) | 15 | 1.29 (1.14, 1.45) | |
| Picloram | 11 | 12 | 1.28 (1.06, 1.56) | 13 | 1.21 (1.09, 1.36) | |
| Amide | | | | | | |
| Dimethenamid | 3 | 3 | 0.91 (0.63, 1.30) | 4 | 1.14 (0.94, 1.37) | |
| Fomesafen | 3 | 3 | 0.98 (0.68, 1.43) | 3 | 1.17 (0.96, 1.43) | |
| Anilide | | | | | | |
| Sulfentrazone | 2 | 2 | 0.76 (0.49, 1.17) | 2 | 0.92 (0.71, 1.20) | |
| Benzoylcyclohexadione | | | | | | |
| Mesotrione | 9 | 10 | 1.17 (0.95, 1.45) | 11 | 1.16 (1.03, 1.31) | |
| Chloracetanilide herbicide | | | | | | |
| Acetochlor | 11 | 10 | 1.00 (0.81, 1.23) | 14 | 1.24 (1.11, 1.39) | |
| Alachlor | 2 | 2 | 1.02 (0.68, 1.54) | 2 | 1.25 (0.99, 1.58) | |
| Metoachlor | 11 | 12 | 1.12 (0.91, 1.37) | 13 | 1.14 (1.01, 1.28) | |
| Cyclohexene oxime | | | | | | |
| Clethodim | 2 | 2 | 1.12 (0.76, 1.66) | 2 | 1.00 (0.79, 1.26) | |
| Sethoxydim | 2 | 3 | 1.18 (0.81, 1.74) | 2 | 0.80 (0.60, 1.05) | 0.076 |
| Dicarboximide | | | | | | |
| Flumioxazin | 1 | 1 | 1.01 (0.54, 1.91) | 1 | 1.03 (0.71, 1.50) | |
| Dinitroaniline | | | | | | |
| Pendimethalin | 6 | 7 | 1.07 (0.84, 1.36) | 7 | 1.05 (0.91, 1.22) | |
| Trifluralin | 7 | 9 | 1.54 (1.22, 1.94) | 9 | 1.24 (1.08, 1.43) | 0.089 |
| Imidazolinone | | | | | | |
| Imazapyr | 1 | 1 | 0.60 (0.27, 1.33) | 1 | 0.92 (0.62, 1.36) | |
| Imazaquin | 2 | 2 | 1.39 (0.95, 2.05) | 3 | 1.37 (1.09, 1.71) | |
| Imazethapyr | 4 | 3 | 1.15 (0.81, 1.62) | 4 | 1.09 (0.90, 1.32) | |
| Nitrile | | | | | | |
| Bromoxynil | 3 | 3 | 1.31 (0.93, 1.85) | 3 | 1.12 (0.92, 1.37) | |
| Nitrophenyl ether | | | | | | |
| Acifluorfen | 1 | 2 | 1.49 (0.97, 2.28) | 2 | 1.14 (0.85, 1.54) | |
| Lactofen | 1 | 1 | 1.53 (0.85, 2.75) | 1 | 1.36 (0.95, 1.97) | |
| Organophosphorous | | | | | | |
| Glufosinate ammonium | 7 | 7 | 1.05 (0.82, 1.33) | 9 | 1.11 (0.97, 1.27) | |
| Glyphosate | 56 | 62 | 1.56 (1.19, 2.03) | 61 | 1.24 (1.07, 1.44) | 0.120 |
| Oxazole | | | | | | |
| Isoxaflutole | 4 | 4 | 0.80 (0.58, 1.11) | 5 | 0.97 (0.82, 1.15) | |
| Plant growth regulator | | | | | | |
| Flumetralin | 1 | 1 | 0.89 (0.52, 1.53) | 1 | 0.93 (0.64, 1.35) | |
| Maleic hydrazide | 2 | 2 | 0.93 (0.62, 1.40) | 2 | 0.89 (0.66, 1.20) | |
| Petroleum products | | | | | | |
| Fatty alcohols | 1 | 2 | 1.00 (0.61, 1.64) | 1 | 0.97 (0.69, 1.38) | |
| Petroleum distillates | 1 | 2 | 2.45 (1.50, 4.03) | 1 | 1.61 (1.15, 2.25) | 0.123 |
| Phenoxy herbicides | | | | | | |
| 2,4-D | 42 | 45 | 1.46 (1.19, 1.79) | 47 | 1.12 (0.99, 1.26) | 0.019 |
| Fluazifop-butyl/fenoxaprop-p-ethyl | 3 | 2 | 0.87 (0.58, 1.29) | 4 | 1.25 (1.03, 1.53) | 0.079 |
| MCPP | 0 | 0 | 0.89 (0.35, 2.29) | 1 | 1.33 (0.83, 2.14) | |
| Pyridine | | | | | | |
| Triclopyr | 5 | 7 | 1.40 (1.11, 1.76) | 6 | 1.16 (1.00, 1.35) | 0.157 |
| Quaternary ammonium | | | | | | |
| Paraquat | 3 | 4 | 1.10 (0.79, 1.55) | 2 | 0.91 (0.71, 1.16) | |
| Sulfonanilide | | | | | | |
| Cloransulam-methyl | 2 | 3 | 0.90 (0.62, 1.33) | 3 | 1.06 (0.85, 1.31) | |
| Triazine | | | | | | |
| Atrazine | 27 | 28 | 1.33 (1.09, 1.61) | 33 | 1.42 (1.26, 1.59) | |
| Simazine | 1 | 3 | 1.71 (1.17, 2.50) | 1 | 0.94 (0.68, 1.28) | 0.008 |
| Triazinone | | | | | | |
| Metribuzin | 1 | 2 | 1.50 (0.97, 2.30) | 2 | 1.23 (0.93, 1.62) | |
| Urea substitute herbicide | | | | | | |
| Chlorimuron-ethyl | 2 | 2 | 1.08 (0.72, 1.64) | 2 | 1.19 (0.93, 1.52) | |
| Diflufenzopyr | 1 | 1 | 1.28 (0.76, 2.17) | 2 | 1.31 (0.97, 1.75) | |
| Metsulfuron-methyl | 1 | 1 | 0.79 (0.43, 1.47) | 1 | 1.09 (0.78, 1.53) | |
| Nicosulfuron | 7 | 7 | 1.05 (0.83, 1.33) | 9 | 1.14 (1.00, 1.30) | |
| Rimsulfuron | 4 | 4 | 1.02 (0.75, 1.40) | 5 | 1.12 (0.94, 1.33) | |
| Thifensulfuron-methyl | 2 | 2 | 0.94 (0.59, 1.50) | 2 | 1.15 (0.88, 1.50) | |
| Other herbicides | | | | | | |
| Bentazon | 2 | 3 | 1.56 (1.06, 2.30) | 2 | 1.03 (0.78, 1.35) | 0.058 |
| Clomazone | 1 | 1 | 0.58 (0.33, 1.00) | 1 | 0.62 (0.43, 0.88) | |

Note: All models adjusted for BMI, current asthma, age, smoking status, state, days applied pesticides, and days drove diesel tractors. Referent group never used that pesticide. All pesticides were classified based on http://www.alanwood.net/pesticides. Flumetsulam is a sulfonanilide herbicide.
*$p$-Values for contrast presented for values < 0.2.

other respiratory outcome. In our sample of 22,134 male farmers from the AHS cohort, we included 78 currently used individual pesticides, 51 of which had not been previously analyzed for respiratory outcomes. These pesticides included some used solely for agricultural purposes (e.g., paraquat) as well as some with residential and public health uses in addition to agriculture (e.g., glyphosate, 2,4-D, permethrin). We also considered allergic and non-allergic wheeze separately because these outcomes may have different etiologies; our previous analyses may have masked associations with allergic wheeze given its lower prevalence. Overall, 29 pesticides had some association with at least one type of wheeze; 19 were significantly associated with allergic wheeze and 21were associated with non-allergic wheeze; 11 pesticides were significantly associated with both. These associations remained when we excluded asthmatics and when we adjusted for correlated pesticides. Exposure–response analyses provided additional evidence for some pesticides, although many of these chemicals were used infrequently making it difficult to evaluate a quantitative relationship. While non-allergic wheeze was three times more common than allergic wheeze, the associations with pesticides were generally of greater magnitude with allergic wheeze. This observation could suggest that individuals with allergic wheeze are more responsive to the environment; which is consistent with animal models that suggest the respiratory impact of some pesticides is stronger in those with allergy (Dong et al. 1998; Proskocil et al. 2008).

In comparing these cross-sectional results to our previous cross-sectional work among farmers (Hoppin et al. 2002a) and commercial pesticide applicators (Hoppin et al. 2006), we see generally similar findings for the chemicals included in these analyses. This sample is similar, but not identical, to our 2002 analysis: 56% ($n$ = 12,331) of the current participants provided information on wheeze at enrollment, while 44% did not. It is possible that some individuals most affected by pesticides chose not to complete the most recent interview, however, it is unlikely to influence our overall findings, and in fact, the prevalence of wheeze is slightly higher in the current analysis (24%) than in the 2002 analysis (21%). Furthermore, this analysis, as was the first, is cross-sectional based on current exposures and current wheeze, so loss to follow-up due to pesticide use at enrollment is not likely to have an impact on the validity of these results, although generalizability to the entire cohort may be affected if persons who were more sensitive to pesticides were more likely to have dropped out.

From the first analysis in 2002 to the current analysis, the prevalence of use of many of the chemicals has decreased. Only glyphosate use increased from the 1993–1997 time frame. Of the 16 herbicides that we evaluated previously, three [butylate, cyanazine, and *S*-ethyl dipropylthiocarbamate (EPTC)] were not evaluated here due to infrequent use. The largest difference between the 2002 paper (Hoppin et al. 2002a) and the current analysis, is the strong positive findings here for 2,4-D and allergic wheeze. When we previously analyzed wheeze as a

**Table 3.** Current use of insecticides and odds ratios for allergic and non-allergic wheeze among 22,134 male participants in the Agricultural Health Study, 2005–2010.

| | Controls ($n$ = 16,885) | Allergic wheeze ($n$ = 1,310) | | Non-allergic wheeze ($n$ = 3,939) | | |
|---|---|---|---|---|---|---|
| Chemical name and group | Current users % | Current users % | OR (95% CI) | Current users % | OR (95% CI) | $p$-Value contrast |
| Biological | | | | | | |
| Bacillus Thuringiensis | 1 | 1 | 1.04 (0.60, 1.80) | 1 | 0.91 (0.61, 1.34) | |
| Carbamate | | | | | | |
| Aldicarb | 1 | 2 | 1.07 (0.68, 1.69) | 1 | 0.75 (0.52, 1.08) | 0.189 |
| Carbaryl | 6 | 8 | 1.70 (1.32, 2.19) | 5 | 1.03 (0.87, 1.22) | 0.001 |
| Carbofuran | 1 | 1 | 1.19 (0.71, 2.00) | 1 | 0.98 (0.70, 1.36) | |
| Neonicotinoid | | | | | | |
| Imidacloprid | 1 | 1 | 0.78 (0.44, 1.38) | 1 | 1.27 (0.91, 1.76) | 0.118 |
| Organochlorine | | | | | | |
| Endosulfan | 1 | 1 | 1.01 (0.57, 1.79) | 1 | 0.89 (0.59, 1.35) | |
| Lindane | 1 | 1 | 1.58 (0.93, 2.70) | 1 | 0.93 (0.63, 1.38) | 0.087 |
| Organophosphorous | | | | | | |
| Acephate | 5 | 6 | 0.84 (0.64, 1.10) | 5 | 0.85 (0.70, 1.02) | |
| Chlorpyrifos | 9 | 11 | 1.23 (1.00, 1.52) | 10 | 1.09 (0.96, 1.24) | |
| Diazinon | 2 | 2 | 1.31 (0.86, 1.98) | 2 | 0.93 (0.70, 1.24) | 0.151 |
| Dimethoate | 1 | 2 | 1.67 (1.03, 2.73) | 1 | 0.73 (0.46, 1.15) | 0.008 |
| Disulfoton | 1 | 2 | 1.17 (0.73, 1.87) | 1 | 0.63 (0.42, 0.95) | 0.037 |
| Malathion | 11 | 12 | 1.48 (1.19, 1.86) | 13 | 1.29 (1.13, 1.46) | |
| Phosmet | 2 | 2 | 1.21 (0.78, 1.90) | 2 | 1.26 (0.97, 1.63) | |
| Tebupirimfos | 5 | 4 | 0.85 (0.63, 1.15) | 6 | 1.13 (0.97, 1.31) | 0.082 |
| Terbufos | 2 | 3 | 1.16 (0.81, 1.68) | 3 | 1.10 (0.87, 1.37) | |
| Pyrethroid | | | | | | |
| Bifenthrin | 1 | 1 | 0.99 (0.54, 1.78) | 1 | 1.06 (0.75, 1.51) | |
| Cyfluthrin | 8 | 8 | 1.02 (0.81, 1.28) | 9 | 1.13 (1.00, 1.29) | |
| Esfenvalerate | 2 | 2 | 0.94 (0.61, 1.46) | 2 | 0.99 (0.76, 1.31) | |
| Lambda Cyhalothrin | 4 | 5 | 1.19 (0.89, 1.59) | 4 | 1.05 (0.88, 1.26) | |
| Permethrin | 6 | 7 | 1.38 (1.09, 1.75) | 8 | 1.35 (1.17, 1.55) | |
| Pyrethins | 1 | 2 | 1.70 (1.13, 2.56) | 2 | 1.43 (1.10, 1.85) | |
| Tefluthrin | 3 | 3 | 1.09 (0.78, 1.52) | 4 | 1.03 (0.86, 1.25) | |
| Zeta Cypermethrin | 1 | 2 | 2.02 (1.24, 3.30) | 1 | 0.88 (0.60, 1.30) | 0.005 |
| Other | | | | | | |
| Fly spray | 1 | 2 | 1.17 (0.72, 1.89) | 2 | 1.43 (1.10, 1.86) | |

Note: All models adjusted for BMI, current asthma, age, smoking status, state, days applied pesticides, and days drove diesel tractors. Referent group never used that chemical. All pesticides were classified based on http://www.alanwood.net/pesticides.
$p$-Values for contrast presented for $p < 0.2$.

**Table 4.** Current use of fumigants, fungicides, and rodenticides and odds ratios for allergic and non-allergic wheeze among 22,134 male participants in the Agricultural Health Study, 2005–2010.

| | Controls ($n$ = 16,885) | Allergic wheeze ($n$ = 1,310) | | Non-allergic wheeze ($n$ = 3,939) | | |
|---|---|---|---|---|---|---|
| Chemical name and group | Current users % | Current users % | OR (95% CI) | Current users % | OR (95% CI) | $p$-Value contrast |
| Fumigant | | | | | | |
| Chloropicrin | 1 | 2 | 1.00 (0.60, 1.67) | 1 | 0.93 (0.64, 1.34) | |
| Fungicides | | | | | | |
| Captan | 2 | 2 | 1.20 (0.80, 1.81) | 2 | 1.00 (0.76, 1.32) | |
| Chlorothalonil | 2 | 2 | 0.96 (0.64, 1.43) | 2 | 0.81 (0.61, 1.08) | |
| Mancozeb | 1 | 1 | 0.93 (0.52, 1.68) | 1 | 1.11 (0.77, 1.61) | |
| Metalaxyl | 2 | 2 | 0.74 (0.46, 1.18) | 1 | 0.84 (0.63, 1.14) | |
| Propioconazole | 1 | 1 | 0.96 (0.52, 1.79) | 1 | 0.68 (0.43, 1.06) | |
| Pyraclostrobin | 2 | 3 | 1.46 (0.99, 2.14) | 2 | 0.94 (0.72, 1.23) | 0.045 |
| Rodenticide | | | | | | |
| Warfarin | 2 | 2 | 1.55 (1.04, 2.30) | 2 | 1.26 (0.98, 1.62) | |

Note: All models adjusted for BMI, current asthma, age, smoking status, state, days applied pesticides, and days drove diesel tractors. Referent group never used that pesticide. All pesticides were classified based on http://www.alanwood.net/pesticides.
$p$-Value for contrast presented for values < 0.2.

single outcome, we saw no association for 2,4-D and wheeze, but when we stratified on allergic status, we saw an association that was limited to allergic wheeze as well as evidence of an exposure–response relationship. We also saw associations of dicamba with both allergic and non-allergic wheeze in the current analysis that we did not see for wheeze overall previously. We continued to see associations with atrazine, glyphosate, trifluralin, and petroleum oil. We no longer observed an association with paraquat; a chemical for which use has declined over the 10-year period of AHS data collection. For insecticides, we evaluated nine of the previous 15, and continued to see associations for permethrin, carbaryl, and malathion. We previously observed associations with chlorpyrifos use, particularly among commercial pesticide applicators (Hoppin et al. 2006), that we did not see here, but frequency of chlorpyrifos has decreased dramatically since 1993–1997. At that time, 38% of the cohort was applying chlorpyrifos 5 or more days a year; for this analysis, only 3% were applying 6 or more days/year (the top third). None of the fungicides that we evaluated previously were associated with wheeze earlier or in the current analysis.

The three most commonly used herbicides—glyphosate, 2,4-D, and atrazine—were associated with wheeze with evidence of an exposure–response relationship for at least one of the wheeze outcomes; correlations among these pesticides did not explain these findings. Glyphosate and atrazine were associated with both allergic and non-allergic wheeze, whereas 2,4-D was primarily associated with allergic wheeze. Use of glyphosate, commonly sold under the trade name Roundup®, has increased dramatically since our initial analysis due to the widespread use of Roundup® Ready corn and soybean seed. The percentage of farmers applying glyphosate 20 or more days/year increased from 5% to 11% between enrollment (1993–1997) and the current interview (2005–2010). With this increased frequency of use, we had more power to detect associations; but the effect estimates were very similar to those reported previously. Animal studies have suggested a potential mechanism for glyphosate-induced airway inflammation; glyphosate exposures increased eosinophil and neutrophil counts, mast cell degranulation, and production of the cytokines IL-33, TSLP, IL-13, and IL-5; co-administration of ovalbumin did not change the inflammatory immune response (Kumar et al. 2014). Glyphosate was also associated with rhinitis among both farmers and commercial applicators in the AHS (Slager et al. 2009, 2010). In commercial applicators, 2,4-D was associated with rhinitis only when used with glyphosate; we did not see similar evidence of an interaction for either type of wheeze, but the farmers evaluated here use chemicals much less frequently than do commercial applicators. In asthma analyses in the AHS, 2,4-D was significantly associated with allergic, but not non-allergic asthma among women (Hoppin et al. 2008), and with a monotonic increase in allergic asthma prevalence among male farmers (Hoppin et al. 2009). In animal studies, 2,4-D has been associated with respiratory allergy in mice (Cushman and Street 1982) and with sensitization and subsequent respiratory IgE allergic response in mice (Fukuyama et al. 2009). While we continued to see an association of wheeze with atrazine use, animal data to evaluate this finding are limited. In our earlier analysis of commercial pesticide applicators (Hoppin et al. 2006), the association between atrazine and wheeze was confounded by chlorimuron-ethyl, a chemical not commonly used by farmers.

Organophosphate and carbamate insecticides have been associated with wheeze and asthma in this (Hoppin et al. 2002a, 2006, 2008, 2009) and other populations (Fieten et al. 2009; Raanan et al. 2015; Senthilselvan et al. 1992; Yemaneberhan et al. 1997). Here, we saw diminished evidence for an association between respiratory symptoms and use of organophosphate insecticides, although malathion continued to be associated both with allergic and non-allergic wheeze. Malathion was associated with increased skin-prick test sensitization to *Dermatophagoides pteronyssinus* in an Ethiopian study (Yemaneberhan et al. 1997). Chlorpyrifos was associated with allergic wheeze with increased use, but the exposure–response results were not strong. In animal studies, organophosphate insecticides contribute to airway hypersensitivity through the decreasing M2 muscarinic receptor responsiveness and not through acetylcholinesterase inhibition (Fryer et al. 2004); moreover, allergen-sensitized animals are more responsive (Proskocil et al. 2008). To date, animal models have been used to evaluate parathion, diazinon, and chlorpyrifos; whether malathion may also act through these mechanisms is not known.

Carbaryl, a carbamate insecticide sold under the trade name Sevin, was significantly associated with allergic wheeze with strong evidence of an exposure–response relationship. Carbaryl was the third most commonly used pesticide in the home and garden sector and the most commonly used insecticide in 2007 (U.S. EPA 2011a). Carbamate insecticides as a group, and carbofuran in particular, were associated with self-reported asthma among farmers in Saskatchewan, Canada (Senthilselvan et al. 1992). In rats, carbaryl enhanced the pulmonary allergic responsiveness to house dust mite (Dong et al. 1998) and has been shown to alter the Th1/Th2 balance in developing rats (Jorsaraei et al. 2014).

Pyrethroids are among the most commonly used insecticides, particularly for residential uses. In 2007, pyrethroids as a group were the second most commonly used insecticide in home and garden settings (U.S. EPA 2011a). All the pyrethroids that we evaluated, except tefluthrin, are approved for residential uses (U.S. EPA 2011b). Three of the eight pyrethroid insecticides were associated with wheeze. Cyfluthrin, the most commonly used pyrethroid in this study, was not. The most frequent users of permethrin were more likely to report wheeze, both allergic and non-allergic. Other investigators have found suggestive evidence of wheeze associated with permethrin exposure among children (Liu et al. 2012). Pyrethroids as a group may influence production of the cytokine IL-10 (Neta et al. 2011) and interferon-gamma and

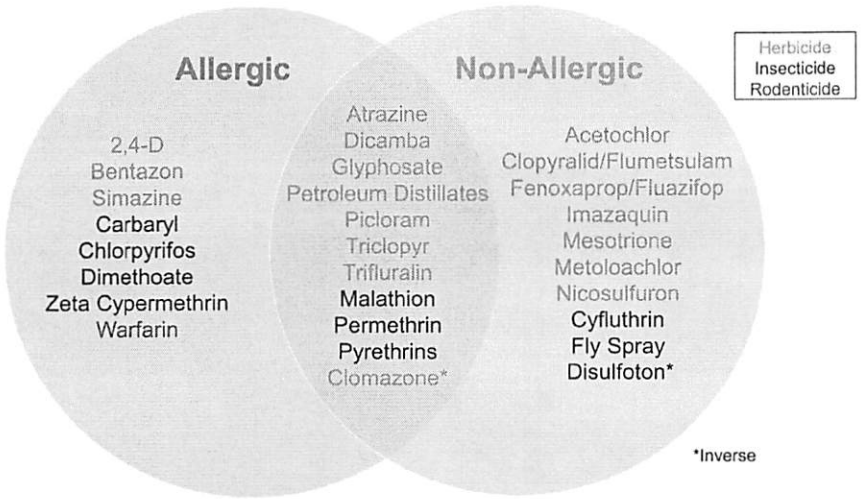


**Figure 1.** Current use pesticides associated with allergic and non-allergic wheeze among the 78 evaluated.

IL-4 (Diel et al. 2003) in humans, but studies are limited. In 2009, the U.S. Environmental Protection Agency (EPA) reviewed the relationship between pyrethroid exposure and asthma and allergy and found "no clear or consistent pattern of effects reported to indicate conclusively whether there is an association between pyrethrins/pyrethroid exposure and asthma and allergy;" however, all pesticide registrants were asked to provide the U.S. EPA with detailed follow-up of reported pyrethrins incident cases (U.S. EPA 2009).

The rodenticide warfarin was strongly associated with increased allergic wheeze. Some of this increase is possibly due to the environments in which rodenticides are used on farms (barns, silos) which may result in increased allergen exposure. Warfarin is also a commonly used anti-coagulation agent in human medicine. Wheezing is one of the rarer reported side effects (http://www.drugs.com/sfx/warfarin-side-effects.html) and allergic reactions are considered an even more serious side effect.

In conducting this analysis, we did not adjust for multiple comparisons nor did we undertake analysis of potential mixtures. Other authors (Goldberg and Silbergeld 2011; Rothman 1990) have argued against adjusting for multiple comparisons with preference for data presentation consistent with Bradford–Hill criteria (Hill 1965), where study design, analytical approach, and consistency of the evidence over time or across study populations are given priority. Even in a sample as large as ours, analysis of potential combinations of agents is challenging, particularly when most chemicals are used fewer than 5 days a year. Overall the effect estimates were generally low (ORs ranging 1.13–2.45) which is an advantage of our large sample size, but suggests that residual confounding may explain our associations. We did use statistical measures to minimize potential confounding by including a covariate for overall pesticide use in the growing seasons and frequency of driving diesel tractors. We grouped pesticides that came from the same technical product, as neither we nor the users are able to separate out exposure to the individual chemicals. We saw more statistical associations than would be expected by chance (5% of 156 comparisons equals eight expected significant chemicals where we observed 40 significant); and, for a few chemicals, limited laboratory evidence supports these associations. The lack of comprehensive toxicological testing for respiratory and allergic hazards presents a challenge in interpreting our findings. One issue in interpretation is the lack of ability to evaluate the other ingredients in these pesticides because this information is not publicly available (U.S. EPA 2003; Petrelli et al. 1993). It is possible that some common other ingredient

**Table 5.** Selected exposure–response models for pesticides and allergic and non-allergic wheeze among 22,134 male farmers in the Agricultural Health Study (2005–2010).

| | | Controls ($n$ = 16,885) | Allergic wheeze ($n$ = 1,310) | | Non-allergic wheeze ($n$ = 3,939) | |
|---|---|---|---|---|---|---|
| Chemical | Days/year used | Current users % | Current users % | OR (95% CI) | Current users % | OR (95% CI) |
| Herbicides | | | | | | |
| 2,4-D | Never | 17 | 15 | 1.00 | 16 | 1.00 |
| | Past | 40 | 39 | 1.23 (1.03, 1.48) | 37 | 0.96 (0.86, 1.07) |
| | 1–3 | 16 | 16 | 1.34 (1.06, 1.71) | 17 | 1.10 (0.96, 1.27) |
| | 4–7 | 13 | 13 | 1.41 (1.10, 1.81) | 14 | 1.11 (0.96, 1.29) |
| | 8–10 | 6 | 6 | 1.35 (0.99, 1.82) | 7 | 1.07 (0.90, 1.28) |
| | 11–15 | 3 | 4 | 1.84 (1.30, 2.60) | 3 | 1.08 (0.87, 1.35) |
| | 16–365 | 4 | 6 | 1.87 (1.38, 2.55) | 6 | 1.29 (1.07, 1.55) |
| Atrazine | Never | 23 | 22 | 1.00 | 19 | 1.00 |
| | Past | 50 | 50 | 1.29 (1.10, 1.52) | 48 | 1.15 (1.04, 1.27) |
| | 1–4 | 10 | 9 | 1.20 (0.94, 1.54) | 12 | 1.41 (1.23, 1.63) |
| | 5–7 | 8 | 9 | 1.47 (1.13, 1.90) | 9 | 1.35 (1.15, 1.58) |
| | 8–10 | 5 | 5 | 1.40 (1.03, 1.91) | 6 | 1.50 (1.26, 1.80) |
| | 11–14 | 1 | 2 | 1.81 (1.11, 2.94) | 2 | 1.72 (1.28, 2.31) |
| | 15–122 | 3 | 3 | 1.20 (0.83, 1.74) | 4 | 1.39 (1.12, 1.73) |
| Dicamba | Never | 46 | 48 | 1.00 | 42 | 1.00 |
| | Past | 42 | 39 | 1.13 (0.97, 1.32) | 44 | 1.11 (1.01, 1.21) |
| | 1–2 | 3 | 3 | 1.09 (0.76, 1.56) | 4 | 1.25 (1.02, 1.52) |
| | 3–5 | 5 | 5 | 1.30 (0.97, 1.75) | 6 | 1.22 (1.03, 1.45) |
| | 6–7 | 1 | 1 | 1.20 (0.70, 2.06) | 2 | 1.44 (1.08, 1.92) |
| | 8–10 | 1 | 1 | 1.12 (0.66, 1.90) | 2 | 1.42 (1.08, 1.87) |
| | 11–110 | 1 | 2 | 2.00 (1.27, 3.16) | 1 | 1.35 (0.99, 1.85) |
| Glyphosate | Never | 11 | 8 | 1.00 | 9 | 1.00 |
| | Past | 33 | 30 | 1.13 (0.90, 1.43) | 29 | 1.05 (0.92, 1.19) |
| | 1–5 | 20 | 21 | 1.47 (1.11, 1.96) | 22 | 1.23 (1.05, 1.44) |
| | 6–11 | 16 | 17 | 1.57 (1.17, 2.10) | 18 | 1.24 (1.05, 1.46) |
| | 12–15 | 6 | 8 | 1.73 (1.24, 2.44) | 8 | 1.34 (1.10, 1.63) |
| | 16–25 | 7 | 8 | 1.62 (1.15, 2.28) | 8 | 1.24 (1.02, 1.52) |
| | 25–240 | 6 | 8 | 1.79 (1.26, 2.53) | 7 | 1.23 (1.00, 1.51) |
| Insecticides | | | | | | |
| Carbaryl | Never | 41 | 32 | 1.00 | 41 | 1.00 |
| | Past | 53 | 61 | 1.48 (1.29, 1.69) | 54 | 1.08 (1.00, 1.16) |
| | 1–2 | 2 | 2 | 1.45 (0.95, 2.20) | 2 | 1.02 (0.78, 1.34) |
| | 3–6 | 2 | 3 | 2.07 (1.43, 3.01) | 2 | 1.12 (0.86, 1.47) |
| | 7–10 | 1 | 1 | 1.24 (0.71, 2.17) | 1 | 1.00 (0.69, 1.44) |
| | 11–100 | 1 | 1 | 1.55 (0.87, 2.76) | 1 | 0.76 (0.48, 1.20) |
| Chlorpyrifos | Never | 54 | 51 | 1.00 | 53 | 1.00 |
| | Past | 37 | 39 | 1.13 (0.99, 1.28) | 37 | 0.98 (0.91, 1.06) |
| | 1–2 | 3 | 3 | 1.24 (0.87, 1.76) | 3 | 1.05 (0.85, 1.31) |
| | 3–5 | 3 | 4 | 1.12 (0.81, 1.55) | 4 | 1.12 (0.93, 1.35) |
| | 6–8 | 1 | 2 | 1.48 (0.90, 2.45) | 1 | 0.92 (0.65, 1.31) |
| | 9–10 | 1 | 1 | 1.17 (0.64, 2.12) | 1 | 1.31 (0.94, 1.82) |
| | 11–120 | 1 | 1 | 1.29 (0.72, 2.32) | 1 | 1.07 (0.73, 1.55) |
| Cyfluthrin | Never | 91 | 91 | 1.00 | 89 | 1.00 |
| | Past | 1 | 1 | 0.88 (0.51, 1.52) | 2 | 1.19 (0.90, 1.58) |
| | 1–3 | 3 | 3 | 0.99 (0.68, 1.43) | 3 | 1.04 (0.84, 1.29) |
| | 4–6 | 2 | 3 | 1.16 (0.80, 1.68) | 3 | 1.13 (0.90, 1.41) |
| | 7–9 | 1 | 1 | 0.96 (0.51, 1.82) | 1 | 1.25 (0.90, 1.74) |
| | 10–11 | 1 | 1 | 0.98 (0.50, 1.91) | 1 | 1.42 (1.02, 1.98) |
| | 12–200 | 1 | 1 | 0.79 (0.40, 1.54) | 1 | 1.08 (0.76, 1.53) |
| Malathion | Never | 26 | 22 | 1.00 | 24 | 1.00 |
| | Past | 63 | 67 | 1.39 (1.20, 1.61) | 63 | 1.09 (1.00, 1.19) |
| | 1 | 4 | 3 | 0.99 (0.70, 1.42) | 5 | 1.19 (1.00, 1.43) |
| | 2 | 2 | 4 | 1.93 (1.36, 2.75) | 3 | 1.32 (1.06, 1.64) |
| | 3–4 | 1 | 2 | 2.00 (1.30, 3.08) | 2 | 1.34 (1.01, 1.77) |
| | 5–7 | 1 | 2 | 1.85 (1.12, 3.04) | 1 | 1.18 (0.85, 1.64) |
| | 8–100 | 1 | 1 | 1.08 (0.60, 1.95) | 2 | 1.57 (1.16, 2.12) |
| Permethrin | Never | 73 | 69 | 1.00 | 70 | 1.00 |
| | Past | 22 | 24 | 1.19 (1.03, 1.38) | 22 | 1.05 (0.96, 1.14) |
| | 1–2 | 2 | 2 | 1.31 (0.88, 1.95) | 3 | 1.19 (0.94, 1.50) |
| | 3–6 | 2 | 3 | 1.42 (0.97, 2.09) | 3 | 1.29 (1.02, 1.63) |
| | 7–12 | 1 | 1 | 1.18 (0.67, 2.08) | 1 | 1.52 (1.12, 2.08) |
| | 13–365 | 1 | 1 | 1.79 (1.05, 3.04) | 2 | 1.76 (1.30, 2.39) |
| Rodenticide | | | | | | |
| Warfarin | Never | 97 | 96 | 1.00 | 96 | 1.00 |
| | Past | 1 | 2 | 1.53 (0.99, 2.38) | 2 | 1.33 (1.02, 1.72) |
| | 1–2 | 0 | 1 | 1.50 (0.75, 3.01) | 1 | 1.06 (0.65, 1.71) |
| | 3–6 | 1 | 1 | 1.40 (0.71, 2.74) | 1 | 1.56 (1.07, 2.29) |
| | 7–260 | 1 | 1 | 1.81 (0.92, 3.56) | 1 | 1.07 (0.68, 1.71) |

Note: All models adjusted for BMI, current asthma, age, smoking status, state, days applied pesticides, and days drove diesel tractors. Referent group never used that chemical. Categories are tertiles of the exposure distribution with the top tertile cut in half or thirds, depending on frequency of use of chemical in the past year. Some percentages do not sum to 100 due to rounding.

could explain these associations for specific pesticides, but we are not able to evaluate that using our data. The fact that associations differed by pesticide argues that systematic bias does not explain these results.

Evaluating the respiratory outcomes associated with pesticides is challenging and requires a large number of individuals who are able to provide detailed information regarding their personal pesticide usage. The AHS has both these strengths. Pesticide use history in the AHS is detailed and has been demonstrated to be reproducible (Blair et al. 2002) and accurate (Hoppin et al. 2002b). We evaluated the common respiratory symptom, wheeze, which by nature is a self-reported outcome. Questionnaire assessment of wheeze has been demonstrated to be reliable and reproducible (Burney et al. 1989). Because our study relied on self-reported symptom and pesticide use in the same interview cycle, we cannot be certain that pesticide use preceded respiratory symptoms; this feature also limited our exposure–response analysis. By restricting our investigation to currently used pesticides, we have done our best to ensure that these pesticides were used in roughly the same time window as when wheeze was assessed. It is unlikely that recall bias influenced these findings; not all pesticides were associated with wheeze and we observed differential associations for allergic and non-allergic wheeze. Even if farmers were aware of our previous findings, recall bias could not explain the differential findings for allergic and non-allergic wheeze or the findings for some, but not all, new chemicals. When we limited our analysis to participants without asthma, we saw essentially the same results. Because people may stop using a chemical that triggers respiratory symptoms, we created a separate category for past use of the chemical, so that the referent group was never users of the chemical. For some chemicals, past users had higher odds of wheeze than never users. However, we do not have the data to evaluate whether this observation is an indication of a lingering impact of use or some other exposure. For every chemical, current users at some exposure level had ORs higher than former users.

This is the most comprehensive analysis of current use pesticides and the common respiratory symptom wheeze to date. Our analysis included the majority of pesticides used in agriculture, home and garden, and industrial/commercial/governmental uses in the United States (U.S. EPA 2011a). Our sample includes nine of the 10 most commonly used pesticides in the home and garden sector; six of 10 in the industry/commercial/government sector; and 16 of the 25 in the agricultural market sector (U.S. EPA 2011a). Therefore, our analysis is a good representation of the pesticides used in the United States. While this analysis was limited to male farmers who, most likely, have applied pesticides for decades, the chemicals that they use are not exclusively agricultural. The findings for chemicals like glyphosate, 2,4-D, carbaryl, and the pyrethroids are particularly relevant for consumers who would like to minimize their wheeze and allergy risk associated with the use of chemicals in their homes, gardens and play areas. While 29 of the 78 pesticides showed some association with wheeze, the majority did not. Future studies should focus on potential mechanisms as well as strategies to minimize exposure.

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