BIOMARKERS OF PESTICIDE TOXICITY AMONG TEEN FARMWORKERS

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LIST OF ABBREVIATIONS

BARS	Behavioral Assessment and Research Systems
CI	Confidence Interval
CPT	Continuous Performance
7,8-dihydro-8-oxo-2`	Deoxyguanosine (8-oxodG)
DAP	Dialkylphosphate
DETP	Dielthythiophosphate
DEP	Diethylphosphate
DST	Digit Span Test
DMDTP	Dimethyidithiophosphate
DMSO	Dimethyl Sulfoxide
DMP	Dimethylphosphate
DMTP	Dimethyltlophosphate
ESL	English as a second language
GC/MS	Gas chromatography / mass spectrometry
HPLC-EC	High-performance liquid chromatography with electrochemical detection
MEP	Migrant Education Program
MDL	Minimal Detection Level
NAWS	National Agricultural Workers Survey
NIOSH	National Institute for Occupational Safety and Health
PRT	Progressive Ratio
OPS	Organophosphate pesticides
ROS	Reactive oxygen species
RLT	Reversal Learning
RPMI	Roswell Park Memorial Institute media
SAT	Selective Attention Test
SDL	Serial Digit Learning
SRT test	Simple Reaction time
SDT	Symbol-Digit Test
TAP	Tapping Test
THPI	Tetrahydrophthalimide

ABSTRACT

Adolescents working in agriculture are exposed to pesticide spray, drift, and residues in the soil and on foliage, however little scientific evidence is available to determine acceptable levels of pesticide exposure to this population. Pesticides are thought to pose a considerably higher risk to children than to adults, yet little is known about the extent or magnitude of health problems related to occupational exposure to pesticides in children. It has been suggested that developmental factors- physical, cognitive, and psychological- may place youth workers at increased risk. Currently, handling or applying agricultural chemicals classified under the federal Insecticide, Fungicide, and Rodenticide Act as toxicity category I or II is considered a hazardous work order for youth under the age of 16. However there is no federal youth labor law restricting the handling of category III and IV pesticides. Although certain safety practices are known to protect workers from the acutely harmful health effects of exposure to agricultural chemicals, less is known regarding protection against exposures to low-levels of pesticides, and the association of chronic low-level pesticide exposure and potential neurotoxicity, reproductive toxicity, endocrine disruption, and carcinogenic effects. Some organ systems, such as reproductive and endocrine systems undergo periods of rapid growth and development during adolescence, potentially placing adolescents at an increased risk for long-range chronic or mutagenic effects of these chemicals. Hypothetically, the period of rapid cell growth that occurs during adolescence could increase susceptibility to carcinogens, but little data exist to support or refute this.

The purpose of this project was to evaluate the extent to which adolescent farmworkers differ in their exposure to agricultural chemicals when compared to adult co-workers and to assess differences in the effects of such exposures on measures of DNA damage and neurotoxicity. We compared biomarkers of genetic damage and oxidative stress among adolescents and adults of similar cultural backgrounds and performing similar agricultural work tasks and used neurobehavioral tests to compare performances between adult and adolescent farmworkers.

During two harvesting seasons we recruited 409 Hispanic adolescent and adult farmworkers and controls to participate in the study. All subjects provided urine samples for measures of oxidative stress and for measurement of metabolites of commonly used pesticides. Buccal samples were obtained to measure DNA damage in leukocytes. Subjects completed a neurobehavioral test battery consisting of 10 computer-based tests measuring attention, response speed, coordination and memory.

Using urinary biomarkers of organophosphate pesticides, we found that the exposures of the adolescent and adult farmworkers were similar and that they were not significantly higher than the levels observed in our controls group. Levels of THPI, the metabolite of Captan, a fungicide commonly used in berry crops close to the time of harvest were shifted significantly higher in the agricultural workers relative to the controls (1-sided p-value = 0.01; Wilcoxon test). Specific tests of various percentiles (median, 60th, and 75th percentile) indicated that while medians did not differ in these two populations (1-sided p-value = 0.91), the 60th and 75th percentiles were both significantly higher in the agricultural population (60th percentile, 1-sided p-value = 0.01; 75^{th} percentile, 1-sided p-value = 0.037). Similar differences were observed during both years of data collection.

Age, gender, school experience, and years working in agriculture all impacted performance on the neurobehavioral tests. Comparison of adult and adolescents did not reveal decreased neurobehavioral performance in adolescents. On several tests the adolescents performed better than adult counterparts. The results of the neurobehavioral tests in subjects who were currently working in agriculture, or with previous agricultural experience indicated that cumulative exposure to low levels of pesticides over many years of agricultural work is associated with neurological impairment as measured by the Match-to-Sample Test. Other measures, Selective Attention, Symbol-Digit, and Reaction Time, showed an interaction with years worked in agriculture and gender. Experience handling pesticides was also associated with deficits in neurobehavioral performance on four neurobehavioral measures. Scores on Digit Span forward and Digit Span reverse were significantly lower for men who had handled pesticides (0.51 points lower for forward, $p = 0.02$ and 0.52 points lower for reverse, $p = 0.02$). Match-to-Sample scores were also lower (2.04 points) for men who reported handling pesticides in the past compared to men who had never reported handling pesticides ($p = 0.02$). The percentage of hits on the Continuous Performance test also showed a decrease for men who handled pesticides (6.4 percentage points, $p = 0.047$).

Our results indicate an association between exposure to agricultural pesticides and markers of DNA damage in the participants of this study, with comparable levels of damage in both adolescent and adult workers. The mean comet tail intensity and tail moment was significantly greater for agricultural workers compared to controls (1-sided p-values < 0.001). No comet parameter was significantly associated with years spent working in agriculture or age of the farmworker controlling for potential confounding factors. Comet

analysis of leukocytes from buccal cell offers a non-intrusive method of assessment of DNA among working populations; however, we encountered methodological challenges in cryopreservation of the samples. Cryopreservation decreases the number of viable cells available upon thawing. Comparison of frozen and fresh samples from the same individuals indicated higher viability in fresh samples, but similar group means for comet parameters. The intravariability of comet results do appear to increase with cryopreservation.

In summary we found indications of very low pesticide exposures among the farmworkers in our study, and no significant differences between adolescents and adults. Surprisingly, even with these low exposures we found that farmworkers performed poorer than non-agricultural participants. A substantial proportion of our sample reported previously mixing or applying pesticides and neurobehavioral performance in this subsample appears to be affected with lower performance. On a number of tests cumulative years of farmwork appears to be related to neurobehavioral performance. The findings of significantly increased indicators of DNA damage among the farmworker participants is also of concern given the postulated relationship between DNA damage and subsequent development of a number of chronic disease and cancer.

Highlights/Significant Findings

Levels of the major dialkyphosphate metabolite (DMTP) among teens working in agriculture in 2004 were shifted slightly higher compared to agricultural adults, though not by a significant amount. Exposures to the Captan metabolite as measured by THPI did not differ between adults and teens. The organophosphate pesticide exposures in the study sample were very low and not significantly higher in all of the agricultural subjects combined relative to subjects not working in agriculture. Levels of THPI were shifted significantly higher in the agricultural workers relative to the controls.

The majority of participants completed all of the neurobehavioral tests; however, adult female participants working in agriculture had lower completion rates. We found that adolescents did not have poorer performance on the neurobehavioral test battery and on several tests performed better than the adults.

Performance on several tests decreased as years spent working in agriculture increased. For females, as years working in agriculture increased, performance on the Symbol-Digit and Reaction Time measures decreased. As both age and years of working in agriculture increased in males, performance on the Selective Attention measures decreased.

Any experience of mixing/applying pesticides was found to significantly decrease performance on four neurobehavioral measures (Digit Span forward, Digit Span backward, Match-to-Sample, and the Continuous Performance test). When the subset of participants who had recent experience mixing/applying pesticides was compared to the participants who had no experience handling pesticides, three neurobehavioral measures showed decreased performance.

On the comet assays for DNA damage we found that the mean tail intensity was significantly greater for agricultural workers compared to controls (1-sided p-value < 0.001). Tail moment was also significantly greater for agricultural workers compared to non-agricultural workers (1-sided p-value < 0.001). No comet parameter was significantly associated with years spent working in agriculture (2-sided p-values = 0.40, 0.93, 0.46 for tail length, tail intensity, and tail moment, respectively). Comet parameters were not significantly associated with urinary pesticide metabolites.

There was no indication that adolescent farmworkers had more DNA damage than their adult coworkers. Median tail length and tail moment did not significantly differ between teen and adult agricultural workers. Farmworkers did not have significantly higher levels of the DNA adduct 8-oxodG relative to those individuals not working in agriculture, nor were levels higher in adolescents compared to adults.

Translation of Findings

The results of this study provide evidence that adolescents do not appear to have specific developmental susceptibility to pesticide exposures as measured by neurobehavioral performance and DNA damage. However this study adds to a growing body of evidence that chronic pesticide exposure in farmworkers is associated with effects on neurobehavioral performance. The sources and types of exposures to pesticides in populations who do not mix or handle pesticides needs further attention. Educational programs are needed to communication the results of this work and similar studies. The large proportion of farmworkers who do not speak either English or Spanish is an urgent public health priority. The evidence from this study adds to a growing body of studies on the potential utility of biomonitoring DNA damage and oxidative stress among working populations as an indicator of potential health problems.

Outcomes/Relevance/Impact

This study demonstrates the ability to access a large number of immigrant farmworkers for a scientific investigation on health effects associated with pesticide exposures. The results provide some reassurance of the safety of farmwork for adolescents, but the participants in this study were exposed to very low levels of pesticides, which might not pertain to all types of work experienced by this seasonal and migrant workforce.

The neurobehavioral results add to an increasing body of knowledge of the effect of cumulative years of low level exposure to pesticides on neurobehavioral performance and the alkaline comet results point to the potential utility of biomonitoring farmworkers for cumulative DNA damage and oxidative stress.

SCIENTIFIC REPORT

BACKGROUND

In the U.S. today, an undeterminable number of youth work as hired agricultural labor on a migrant or seasonal basis. Data from the Current Population Survey show that about 116,000 youth ages 15-17 worked in agriculture in 1997 as hired workers. The National Agricultural Workers Survey (NAWS) indicates that about 129,000 14- to 17- year olds work annually as hired farmworkers and make up about 7 percent of all hired farmworkers working on crops(1). However these numbers are most likely underestimates in that they exclude workers under 14 or 15 years of age, and perhaps do not capture the very migrant or undocumented workers. The NAWS data also show a growing proportion of workers between 14 and 17 years of age working away from their parents as unaccompanied minors. In a survey of migrant adolescent farmworkers in Oregon, we previously found that 64.7% of the adolescents living in migrant labor camps were traveling and working in the U.S. unaccompanied by their parents (2). Data documenting the work characteristics of adolescent farmworkers are limited, and there are no published accounts of the particular health hazards of these youth workers. The work exposures of this vulnerable population are significant, with the NAWS indicating that, on average, agricultural workers (ages 14 to 17) work about 31 hours per week and that this youth employment is mainly seasonal.

The special health concerns and research needs of the adolescent migrant population have been addressed by national groups(1,3). While youth labor hours are restricted in other industries, a child under age 16 may generally work in agriculture for an unlimited number of hours as long as the child is not working during school hours. Children under the age of 16 are prohibited from working in various hazardous agricultural occupations. However NIOSH has forwarded even more stringent recommendations to the U.S. Department of Labor for changes to these hazardous orders(4). These recommendations stem in a large part from an enhanced national focus on the safety and health of children.

Researchers have had difficulty in accessing data on special populations such as migrant laborers. Even less information has been available on the health of migrant children, including those who work in agriculture. Using our history of successfully partnering with community organizations that serve migrant youth, engaging this vulnerable population in a variety of research projects, and providing pesticide safety training to this population, we were able to conduct this study focused on biomarkers of vulnerability associated with occupational exposure to pesticides.

Work Patterns/Practices

There are both positive and negative factors associated with child labor. Employment can bring a sense of responsibility, discipline, and teamwork and provide opportunities of the development of new skills (5). Employment opportunities in the United States may provide an opportunity for the migrant adolescent to contribute to the family income and also to continue with education when opportunities are available in the agricultural community. However, migrant adolescent farmworkers are socio-economically disadvantaged and have limited access to medical care. While working in agriculture, these youth may also be exposed to inadequate sanitation, poor nutrition and neglect. Youth associated with farming outside of the traditional family farm are often neglected in efforts to promote health and safety for the typical farm operation in the US.

There is some indication that adolescent farmworkers may be engaged in work practices that put them at increased risk for pesticide exposure. In our previous work, nearly a quarter of the adolescent farmworkers that we surveyed answered affirmatively to the question "In your current job do you mix/apply pesticides, herbicides, fungicides, or other chemicals." Calvert et al. found in survey data from eight U.S. states that the average annual incidence rate for acute occupational pesticide-related illnesses among youths aged 15-17 years was 20.4 per billion hours worked, and the incidence rate ratio of youths vs. adults was 1.71 (95% confidence interval = 1.53, 1.91)(6). The authors speculated that this disparate risk may be attributable to a number of factors such as adolescents being less likely to question work assignments or having less training, or that they may manifest acute illnesses at lower thresholds. Our previous research with this population found pesticide training is lower among adolescents than among adults, and adolescents have reported that they are hesitant to question their "boss" regarding possible exposures to hazardous chemicals. The threshold exposures at which adolescents experience health effects have not been studied among agricultural workers.

Health Effects of Pesticides

Despite the large amounts of pesticide chemicals being used and the potential of many to cause adverse health effects in humans, there are still insufficient data to accurately determine the true impact of pesticides on human health, especially long-term low level exposure to pesticides.

Pesticides as Neurotoxicants: Insecticides designed to attack the insect nervous system (organochlorines, pyrethroids, organophosphous and carbamate esters) are capable of producing acute and chronic neurotoxic effects in humans. Many of the pesticides used by agricultural workers are synaptic transmitters (carbamates, organophosphates); some (organochlorines) cause tremor and seizures, and others (organophosphates) may induce axonal neuropathy. Extrapyramidal and cognitive dysfunction is associated with a number of chemicals that impair energy generation and find use as fungicides, fumigants or rodenticides. Mild psychological and behavioral deficits, such as changes in the speed and precision of answering questions, impaired judgment, poor comprehension, and decreased ability to communicate have been reported to occur after exposure to anticholinesterase pesticides and can persist for weeks to months (7). Chronic effects of pesticide exposure, particularly exposure to organophosphates, are not well characterized and published; studies examining the neurotoxic effects of low-level pesticide exposure to children are limited(8).

Pesticides and Cancer as a Health Endpoint: Numerous studies of agricultural and pesticide workers have reported excess cancers of the hematopoetic system, including multiple myeloma, Hodgkin's lymphoma and non-Hodgkin's lymphoma. More infrequently reported have been soft tissue sarcomas(9), excess cancers of the brain(10), testes(11), bladder (12), squamous cell skin carcinoma(13), pancreas(14,15), oral cavity and pharynx(16), stomach(17), rectum(17), renal (17,18), lung(18) and prostate (19).

Excess non-Hodgkin's lymphoma (NHL) has been associated with pesticide exposure in a variety of studies(20-27) although other studies report no association(28,29). Numerous studies have reported a modest elevation in risk for multiple myeloma among agricultural workers and pesticide applicators(30-32).

Few agricultural workers are exposed to a single, or a single class of, pesticide. Conflicting study results may be explained by the potential for exposure misclassification due to incomplete knowledge of pesticide type, extent of exposure, potential for interactions between mixed pesticides, and differences in personal protection and hygiene. Potential for exposure misclassification is increased in studies of cancers that occur many years after exposure. Measurement of exposures and intermediate health endpoints more proximate to exposure will allow improved exposure estimation and better estimates of associated risk.

Developmental Susceptibility to the Effects of Pesticides

Children working in agriculture are exposed to many of the same occupational hazards as those experienced by adult workers. Only about five percent of farms in this country are covered by safety regulations of the Occupational Safety and Health Act. On the remaining 95 percent of farms, the owner/operator is responsible for assessing acceptable levels of risk for adults and children on the farm. Unfortunately, little scientific evidence is available to determine acceptable levels of hazard exposure to children (33). Standards set up to protect workers are often inappropriate for children and pregnant women. For example, threshold limit values for exposure to various pollutants set up by the National Institute for Occupational Safety and Health (NIOSH) are established for an eight-hour day for adult white men aged 18 to 65. While the standards may be safe for their target population, no one knows the health effects these levels may have on children and the unborn.

Research is needed to document the effects of pesticide exposure in youth workers. Even subtle changes in disease risk among this group of adolescent workers could result in large social and economic consequences for this vulnerable population. In the case of an acute poisoning, estimates of residual disability can be estimated. However it is difficult to estimate the non-life-threatening consequences of either short- or long-term constant or intermittent pesticide exposure. Bellinger argues that limiting concern about the toxic effects of pesticide exposure to end points corresponding to clinical disease might not be appropriate(34). In the case of subtle changes in neurocognitive performance as a consequence of pesticide exposure, the absence of a diagnostic label does not make the exposure-associated deficits any less real or less distressing to a child and family. Likewise, it is important to conduct studies of the risk of DNA damage among youth workers exposed to pesticides to determine if there is any empirical evidence of a developmental vulnerability. While the risk of subsequent development of disease as a result of early DNA damage is unknown and the resulting social and economic consequences of the disease impossible to determine, the information gained from this research advances our understanding of health risks of adolescent workers exposed to occupational chemicals.

Measurement of Biomarkers of Exposure to Pesticides

Biomarkers serve as tools for exploring the effects of environmental exposures. In the last decade, researchers have focused on metabolites of pesticides in the urine of agricultural workers as a sensitive biomarker of exposure. Measurement of urinary metabolites of pesticides are more accurate estimates of internal dose and are particularly useful in this case when multiple routes of exposure are possible (dermal, inhalation, ingestion)(35). Biomarkers of internal dose integrate all pathways of exposure by estimating the amount of pesticide (toxicant) that is absorbed into the body via measurements of the pesticide, its metabolite or its reaction product in biological media (36).

Low-level exposure to organophosphate pesticides can be determined by measurement of a group of six dialkylphosphate (DAP) metabolites in urine. These metabolites are mainly excreted in human urine within 6 to 24 hrs after exposure (37). Nutley and Crocker (37) estimate that almost 80% of the organophosphate pesticides approved for use should yield one or more of these metabolites. The important advantage of these biomarkers is that metabolites of OP pesticides are detectable at exposure levels lower than those that cause depression of cholinesterase (38). Another advantage of urinary metabolites is that a baseline level is not needed, making these biomarkers particularly attractive in studies of migratory farmworker communities where locating and following individuals present distinct challenges.

In addition to measuring the six dialkylphosphate metabolites in urine, pesticide-specific metabolites of organophosphate pesticides can also be measured. The most common metabolite measured is 3, 5, 6-trichlor-2-pyridinol (3, 5, 6-TCPy), a metabolite of chlorpyrifos. Fungicides, although widely used, are not the most common class of pesticides typically measured in humans however methods have been developed to measure metabolites of some of the fungicides including Captan. Captan is measured with its major metabolite tetrahydrophthalimide (THPI).

Pesticides and Neurobehavioral Performance

Research examining neurobehavioral effects of pesticide exposure have focused on pesticide exposures that are the result of poisonings or other acute exposure events (39-41). Extensive research has demonstrated that both acute(42) and chronic(43,44) exposure to neurotoxic solvents, metals, gases and pesticides reduces performance on neurobehavioral tests. The pattern of neurobehavioral deficit following chronic exposures is consistent from study to study(43,44) and it is dose dependent in acute-exposure studies(42). Organophosphate pesticide-poisoned individuals have shown a consistent pattern of deficits when compared to non-exposed or non-poisoned controls on measures of motor speed and coordination, sustained attention, and information processing speed (45, 46)

Similar neurobehavioral tests have been used to study occupational groups chronically exposed to pesticides, including British sheep farmers(47), green house workers(48), tree-fruit workers(49), and Egyptian cotton pesticide applicators(50). These four studies have also found deficits in measures of visiomotor speed and reaction time, and, in addition in the group that may have had the highest exposures, verbal abstraction, attention, and memory(50). Research on neurobehavioral effects of pesticide exposure in children and adolescents is virtually nonexistent.

Pesticides, Oxidative Stress and Genetic Damage

Agricultural workers are generally exposed to a mixture of pesticides, some of which may be capable of causing human cancer, can react directly with DNA, and can induce generation of reactive oxygen species (ROS) that react with DNA. Oxidative damage is thought to be an important mechanism of damage for organophosphate pesticides (OPs)(51,52) and have been reported to generate ROS(53,54) and alter cellular antioxidant systems(55). Pesticide applicators with exposure to commercial grade malathion have been reported in several studies to have excess chromosomal aberrations and sister chromotid exchange (56-58) and Phosmet can cause single strand breaks in human DNA(59) and mutagenic activity in Ames tests(60).

Within the last decade, the comet assay has been used with increasing popularity to investigate the level of DNA damage in biomonitoring studies. (61) The comet assay (single-cell gel electrophoresis) is a rapid and highly sensitive method to detect several types of genotoxic damage including DNA single-strand breaks, alkali-labile sites, and incomplete excision repair sites. Cells with damaged DNA appear as comets with tails due to DNA fragmentation while the DNA of undamaged cells remains intact.

Zeljezic et al. in a study of Croatian pesticide production workers reported that after a period of high exposure to a mixture of pesticides statistically significantly increased levels of DNA damage in the comet assay in terms of tail length and tail moment were found (62). After the workers were removed from production for 8 months, both comet assay end-points decreased significantly compared with the first sampling point, but they remained increased compared with the control. Sailaja et al. conducted the comet assay on peripheral

lymphocytes of pesticide manufacturing workers in India and found significant increases in comet tail length when compared to controls and that neither age nor smoking appeared to be major confounders in the findings (63). Bhalli et al. studied Pakistani pesticide-manufacturing workers and also found that exposed workers had significantly longer tail length compared to controls (64). These findings were similar to those reported by Grover et al. of increased tail length among Indian pesticide production workers (65).

The first report of the comet assay being used to assess DNA damage among agricultural workers was Lebailly et al., who observed significant increases in DNA damage one day after spraying a mixture of pesticides (66). In a more recent report, Lebailly did not observed increases in DNA damage after spraying with the fungicide Captan, which has known mutagenic properties (67). Piperakis et al. conducted biomonitoring with the comet assay of greenhouse workers in Greece exposed year round to pesticides (68). These investigators did not observe any differences in DNA damage between greenhouse workers in Greece and controls. A second report by Piperakis et al. of greenhouse workers in Hungary with subgroups characterized as having symptoms associated with high levels of pesticide exposure also failed to detect any DNA damage associated with pesticide exposure (69). Two other groups of investigators, however in recent years have reported statistically significant increases in DNA damage among pesticide sprayers in Ecuador and Iranian insecticide formulators (70,71).

Measurement of products of oxidative damage in urine reflects overall damage to all tissues and organs in the body. The most studied and abundant oxidation product is the C-8 hydroxylation of the guanine base (8-oxoG), which is measured as the oxidized deoxynucleoside, 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG)(72,73). This DNA adduct, 8-oxodG is highly mutagenic and can accumulate in DNA nuclei and in mitochondria. There have been limited epidemiological studies of the effects of pesticide exposure on the body's production of products of oxidative damage. Tope and Panemangolore studied farmworkers over a 6 month work period. Urinary levels of 8-oxoGd did not differ between the farmworkers and controls, but there was a four-fold increase in the 8-oxodG levels in farmworker serum (74). The correlations between serum or intracellular levels of 8-oxodG and levels found in the urine have not been thoroughly established.

Summary

This study was designed to focus specifically on the question of whether adolescent farmworkers differed from their adult co-workers on a number of workplace exposures and work practices. Biomarkers of exposures that have been commonly used in research investigations of pesticide exposure were used to compare the exposures of the adult and adolescent workers. We used a highly reliable and valid neurobehavioral test batteries to determine if adolescent farmworkers demonstrated an effect on memory, concentration and other measures to a greater extent than their adult counterparts. Finally we used biomarkers of DNA damage and oxidative stress to assess subtle differences between adults and adolescents and between farmworkers and control populations.

SPECIFIC AIMS

Adolescents working in agriculture are exposed to pesticide spray, drift, and residues in the soil and on foliage, however little scientific evidence is available to determine acceptable levels of pesticide exposure to this population. Pesticides are thought to pose a considerably higher risk to children than to adults, yet little is known about the extent or magnitude of health problems related to occupational exposure to pesticides in children. It has been suggest that developmental factors- physical, cognitive, and psychological- may place youth workers at increased risk(75, 76). Currently, handling or applying agricultural chemicals classified under the federal Insecticide, Fungicide, and Rodenticide Act as toxicity category I or II is considered a hazardous work order for youth under the age of 16. However there is no federal youth labor law restricting the handling of category III and IV pesticides. Although certain safety practices are known to protect workers from the acutely harmful health effects of exposure to agricultural chemicals, less is known regarding protection against exposures to low-levels of pesticides, and the association of chronic low-level pesticide exposure and potential neurotoxicity, reproductive toxicity, endocrine disruption, and carcinogenic effects. Some organ systems, such as reproductive and endocrine systems undergo periods of rapid growth and development during adolescence(76), potentially placing adolescents at an increased risk for long-range chronic or mutagenic effects of these chemicals. Hypothetically, the period of rapid cell growth that occurs during adolescence could increase susceptibility to carcinogens, but little data exist to support or refute this(76).

In this award we evaluated the extent to which adolescent farmworkers differ in their exposure to agricultural chemicals when compared to adult co-workers and assessed differences in the effects of such exposures on measures of DNA damage and neurotoxicity. We compared biomarkers of genetic damage and

oxidative stress among adolescents and adults of similar cultural backgrounds and performing similar agricultural work tasks and used neurobehavioral tests to compare performances between adult and adolescent farmworkers. The study was designed to address the following questions:

1. Do short-term biomarkers of exposure to pesticides (including insecticides and fungicides) differ in adolescents employed in agriculture compared to adult farmworkers controlling for type of agricultural work, hours worked, and reported hygiene practices?
2. Is there evidence of a correlation between biomarkers of exposure to organophosphate pesticides and neurobehavioral performance in adolescent farmworkers and is that correlation similar to that observed among adult farmworkers?
3. Is there an association between exposure to agricultural pesticides and markers of DNA damage and oxidative stress in agricultural workers and does this association differ between adult and adolescent farmworkers controlling for work history, hygiene practices and lifestyle factors?

RESEARCH METHODS

We conducted an epidemiological investigation to evaluate the extent to which adolescent farmworkers differ in their exposure to agricultural chemicals when compared to adult co-workers and assessed adult/adolescent differences in the effects of such exposures on neurobehavioral performance and markers of DNA damage. The investigation took place in a highly agricultural region of Oregon dependent on migrant farmworker labor for the harvest of crops. This interdisciplinary research project included investigators with epidemiological, biomarker, exposure assessment, and neurobehavioral scientific expertise. The hypotheses tested included:

1. Do short-term biomarkers of exposure to pesticides (including insecticides and fungicides) differ in adolescents employed in agriculture compared to adult farmworkers controlling for type of agricultural work, hours worked, and reported hygiene practices?
2. Is there evidence of a correlation between biomarkers of exposure to organophosphate pesticides and neurobehavioral performance in adolescent farmworkers and is that correlation similar to that observed among adult farmworkers?
3. Is there an association between exposure to agricultural pesticides and markers of DNA damage and oxidative stress in agricultural workers and does this association differ between adult and adolescent farmworkers controlling for work history, hygiene practices and lifestyle factors?

Targeted Agricultural Work

Agriculture is a vital sector of Oregon's economy. There is a wide diversity of crops reflecting the geographical and climatic diversity within the state. In 2001 more than 27,000,000 harvested acres were devoted to agriculture. The acreage was divided among roughly 40,000 farms, with an average size per farm of 430 acres. For this study we focused on workers harvesting small fruit and berry crops. Nearly 27,000 acres of Oregon farmland were devoted to berry crops and Oregon ranked first, second and third nationwide for the cultivation of varieties of berries, including: blackberries, loganberries and boysenberries, raspberries, and strawberries, respectively (Oregon Agricultural Statistics Service 2001-2002). There are at least seven distinct agricultural growing areas within the state including the 12,000 sq miles of the Willamette River Basin. The Willamette Valley produces nearly 90% of all small fruits and berries grown in the state and nearly 50% of the berries are harvested in Marion and Washington counties. We focused our study recruitment to workers living and working in these two counties. The labor force that harvests the fruit and berries in these counties are highly mobile. Most reside in the community for only the two months of harvesting (June-July) before moving onto other agricultural work, necessitating rapid and efficient methods of data collection.

Target Population and Recruitment

Our agricultural population of interest included adolescent and adult migrant Latino farmworkers in the northern Willamette Valley of Oregon. A comparison group of adolescent and adult Latino individuals who do not work in agriculture was also recruited from the same geographic area. The methods for sample recruitment included:

1. **Recruitment of Adolescent Agricultural Workers in the Oregon Migrant Education Program (MEP).** The student population served by the MEP is approximately 86% Hispanic. To qualify for the Oregon Migrant Education Program, a migrant child must have moved within the past three years across state or school district lines with a migrant parent, guardian, or spouse, or a member of the child's immediate family to obtain temporary or seasonal employment in an agricultural or fishing

activity. The child may be any grade between preschool and grade 12, and between 3-21 years of age, and has not received a high school diploma or GED. A "migrant child" remains eligible for three years after his or her family's last qualifying arrival date. The Hillsboro School District in Washington County and the Marion County School Districts, located in the Willamette Valley, are important home bases for the migrant population and serve a large proportion of the total migrant youth receiving educational services. We proposed to study "migrant youth" eligible for Migrant Education Services and between the ages of 13-18. We also recruited from the Summer Evening Migrant English as a Second Language (ESL) Program that is part of the MEP. This program enrolls approximately 300 students per summer. Many of these enrollees are working in U.S. agriculture and living in migrant labor camps not accompanied by their parents.

2. **Recruitment of Non-Agricultural Adolescent Workers.** We also used the Migrant Education Program in these sites to recruit control Latino youth each year who are eligible for the program (due to their family status), but who are not working in agriculture, nor have in the past. These youth are usually not in the ESL program, but attend the MEP during the school year and are also attending the regular (non-evening ESL) summer school.
3. **Recruitment of Adult Agricultural Workers ages 19-21**. The evening ESL program is open to farmworkers ages 13-21, therefore we were also able to recruit some of our adult farmworker sample from the ESL program.
4. **Recruitment of Adolescent and Adult Farmworkers from Labor Camps.** Our previous studies have provided data that the work experiences of farmworkers enrolled in the evening ESL do not differ significantly from the work experiences of farmworkers not enrolled in ESL. However, we found in 2002 that the pesticide knowledge levels of adolescent farmworkers in the ESL program were higher than adolescents recruited from the labor camps ($t(178)=2.97$, $p=0.003$). No such differences existed between scores of adult from the ESL program compared to adults from the camps. Given that pesticide knowledge could affect work behaviors and protection, we recruited a sample of adult and adolescents (not enrolled in ESL) from the labor camps.
5. **Recruitment of Adults Not Working in Agriculture**. We also recruited a convenience sample of Latino adults ages 19 and older who are immigrants who have not worked in agriculture. These adults were recruited through our contacts at support services serving the Latino population in Oregon.

Recruitment Procedures

Study participants enrolled in the MEP regular school year/summer school program were recruited by preliminary information regarding the research study being given by a Migrant Education staff person during the last quarter of the school year (April-May). This staff person provided information in Spanish on the purpose of the study, the amount and nature of involvement required should the student choose to participate, and the monetary incentives for participating. All Latino migrant youth in the middle and senior high school were approached. Youth who indicated an interest in the study were scheduled for an interview time in which the informed consent process was explained. Study consent forms were sent home to the parents of the youth and follow-up telephone calls were made by bilingual research staff to answer any questions the parents may have about the study. The youth returned the signed parental consent forms before they were enrolled in the project.

Adult and adolescent farmworkers recruited from the ESL Program were given preliminary information regarding the research study by a Migrant Education staff person at the time of enrollment in the evening program. This staff person provided information in Spanish on the purpose of the study, the amount and nature of involvement required should the student choose to participate, and the monetary incentives for participating. All students (ages 13-21) received information on the study. Persons who indicated an interest in the study were scheduled for an interview time in which the study protocol was explained and informed consent forms signed (if 18 or older) or sent with the youth (if traveling with parents) for parental consent. If a youth was under the age of 18 and signed that he/she is traveling and working in agriculture without accompaniment of parents or guardian they were asked to give written indication of this working situation and was allowed to sign the informed consent form.

Adults and adolescent farmworkers recruited in the labor camps were approached in the afternoon hours on the grounds of the camps. Bilingual research staff provided information in Spanish on the purpose of the study, the amount and nature of involvement required should the individual choose to participate, and the monetary incentives for participating.. Persons who indicated an interest in the study were scheduled for an interview time in which the study protocol was explained and informed consent forms signed (if 18 or older) or informed consent was obtained from parents of youth in the case of farmworkers under the age of 18. If a youth was under the age of 18 and signed that he/she is traveling and working in agriculture without

accompaniment of parents or guardian he/she was asked to give written indication of this working situation and was allowed to sign the informed consent form.

We recruited a **convenience sample of Latino adults ages 19 and older** who are immigrants who have not worked in agriculture. These adults were recruited through our contacts at support services serving the Latino population in Oregon. We attended meetings of these community organizations and explained the purpose of the study, the amount and nature of involvement and the monetary incentives for participating. Interested persons were scheduled for a testing/interview session in which informed consent was obtained.

Testing Procedures

During the test session, questionnaires on work histories and work practices associated with reducing pesticide exposure were administered. Short questionnaires on smoking/alcohol and exercise patterns were administered. Weight and height were measured. Each participant completed a computerized test battery designed to detect toxic effects of pesticides on response time, memory and concentration. These tests took approximately one hour for completion. A spot urine sample was obtained for analysis of pesticide biomarkers and biomarkers of oxidative stress. A mouth wash with instruction for rinsing the mouth was administered for buccal rinse epithelial cell analyses. Participants received $25 gift card and a copy of a pesticide safety-training booklet.

Study Instruments

The questionnaire to measure agriculture work was adapted from the "Agricultural Work Practices Questionnaire" that we have used since 1996 in our investigations of pesticide exposure in minority agricultural families. The questionnaire contains items on type of work activity, pesticide application, type of crop(s), hours per week, use of protective clothing, bathing, laundry, and wearing of clothing outside of fields. New sections were added including more detailed work history for the week prior to data collection, short diet and activity history, and brief tobacco/alcohol history.

Specimen Collection and Transport

Buccal Cell Collection: Subjects were given labeled collection cups with 10 ml sterile Hanks' Balanced Salt Solution and were instructed to vigorously rinse their mouth for 60 seconds, then spit into the cup. Collection was monitored and timed by study personnel. Specimens were transported on ice to CROET laboratory facilities. Buccal cell washes were processed within 5 days of collection and cryopreserved. Cryopreservation was as follows: buccal cells were split into 1-ml portions, placed in cryogenic vials, put in an ice-cold medium (40% RPMI 1640, 50% fetal bovine serum and 10% DMSO), cooled at a rate of -1°C/min and stored at –90°C. See the results section for methodological challenges that arose from this cryofreezing procedure.

Urine Collection: Single void (spot) urine samples were collected from the study participants at approximately the same time of day (6-8 PM). Samples were transported on ice to the lab at which time the total volume was recorded. Samples were divided and stored at -80°C in tubes without any additives.

Urinary Analysis for Pesticide Metabolites

The pesticide metabolites that we analyzed in urine samples were determined based on three factors: 1) characteristics of the Oregon berry industry and state guidelines of pesticide use, 2) previous environmental samples in the berry industry communities that have shown the most common agricultural pesticides in home dust, and 3) our laboratory capability. .

Urine samples were analyzed for organophosphate metabolites according to methods of Loewenherz and Fenske (77). Five dialkylphosphates were analyzed by gas chromatography (GC) with pulsed flame photometric detection (PFPD): dimethylphosphate (DMP), diethylphosphate (DEP), dimethylthiophosphate (DMTP), dielthythiophosphate (DETP), and dimethyldithiophosphate (DMDTP). Aliquots of the sample underwent azeotropic distillation, centrifugation, and evaporation under a nitrogen stream, with reconstitution in acetonitrile and derivitization with pentafluorobenzylbromide and heating to convert phosphate acids to esters. The metabolites were confirmed with gas chromatography/mass spectrometry (GC-MS). The method has good precision, excellent recovery, and detection limits around 2-5 µg/L.

In addition to the DAP metabolites, we analyzed all urine samples for Captan (133-06-2, *N*-trichloromethylthio-4-cyclohexene-1,2-dicarboximide, $C_9H_8Cl_3NO_2S$) an organochlorine fungicide of the dicarboximide chemical family, that is used on both apple and berry crops in Oregon. Captan has been classified as a B2 (probable human) carcinogen (78). Previously we had detected Captan in twenty-five percent of the farmworker homes we sampled in ranges from 0.5ppm-19 ppm (79). In that season, Captan had

been applied to berry crops 2-4 times from April through June. Captan was measured as its major urinary metabolite, tetrahydrophthalimide (THPI) using GC-Coulson electroconductivity detection (GC-CECD) with minimum detectable limits of about 5 ppB (80). In brief the analytical procedure used was as follows: urine samples were extracted with Methylene chlorine, dried on a Roto-Vap evaporator, passed through a Selica SepPak cartridge, taken to dryness and reconstituted with Benzene and analyzed on GC/MS in a SIM acquisition mode using m/z 151 and 278.

All urine samples were measured for creatinine concentration (mg %) using the Sigma Diagnostic Creatinine Assay Kit No 555-A and a Spectra MAX 190 plate reader to identify abnormal samples. Samples below the 5th and above the 95th percentiles (mg %) were excluded from the analytical sample to remove very dilute and very concentrated samples with possibly unreliable biomarker measurements.

Analysis of Urine and Buccal Cells for Biomarkers of DNA Damage and Oxidative Stress

Comet Assay of Buccal Cells: The comet assay has become a reliable method for analyzing cells for DNA damage and is based upon the labiality of DNA lesions to alkali. Cells with damaged DNA appear as comets with tails due to DNA fragmentation while the DNA of undamaged cells remains intact. A commercial kit (Comet assay Kit™, Trevigen; Gaithersburg, MD) is available that contains treated slides that promote agarose adherence and a hydrophobic barrier for FLARE treatment. Frozen buccal cells were thawed (quickly by submerging in a 37°C water bath and washing with ice-cold wash medium (40% RPMI, 50% FBS, 10% glucose) and assayed in batches. An aliquot of the thawed cells (~50 cells) were pipetted onto separate slides for analysis by comet. The slides were run in a horizontal electrophoresis apparatus, treated with an alkaline lysis solution, neutralized, treated with 20 µg/ml of Propidium Iodide and the extent of DNA damage determined by examining the cells for tail length and moment using a fluorescence microscope equipped with an automated digital analysis system running comet assay II. The captured images of comets were analyzed for three parameters of DNA damage: tail length; tail fluorescence intensity (percent of DNA in tail) and tail moment (roughly the product of tail length and tail intensity).

Analysis of Urine for Oxidative DNA Damage (8-oxodG): We measured the overall state of oxidative stress in pesticide workers by examining the urine of adult and adolescent farm workers for 8-oxodG by high-performance liquid chromatography with electrochemical detection (HPLC-EC). Frozen urine samples were thawed in a water bath, the samples adjusted to pH 7, centrifuged and 2 ml of the pH adjusted urine loaded onto a preconditioned C18 SFE SepPak™ cartridge (Waters Associates). The SepPak cartridge was washed successively with 25 mM K_2HPO_4 (pH 7.5) and 25 mM K_2HPO_4/5% MeOH buffer, and the samples eluted with K_2PO_4/10 % MeOH buffer. The samples were separated on a Synergi Max-RP HPLC column (250 mm x 4.6 mm x 4.0 µm) connected to a guard column (AJO-6074, 4.0 mm L x 3.0 mm ID) using 25 mM K_2HPO_4 (pH 7.5)/5% MeOH as the mobile phase and 8-oxodG and thymine glycol detected with a LC-4C amperometric detector (BAS, Inc) equipped with a glassy carbon electrode, operating at a working potential of +0.8 V vs. Ag/AgCl reference electrode. Data acquisition was carried out on a PC computer running Mellinnium™ software (Waters Associate). Each sample was run in duplicate, averaged and the results expressed as nmol 8-oxodG/L.

Neurobehavioral Test Battery

Neurobehavioral function was measured with a computerized battery of nine performance tests from the Behavioral Assessment and Research System (BARS) in a 50-minute period. A brief description of each test follows:

1. The Symbol-Digit Test (SDT) is a computer-based adaptation of the Digit-Symbol test from the Wechsler Adult Intelligence Scale (WAIS-R, 11). Nine unique symbols are paired with the numbers one through nine. Participants are shown a matrix that contains only the symbols and asked to press the corresponding numbered button for each pair. Latencies for each button press are recorded.
2. In the Simple Reaction Time (SRT) test, participants are to press a button as quickly as possible when a large square appears on the screen.
3. The Digit Span Test (DST) sequentially presents a series of numbers on the screen, and the subject is asked to reproduce the sequence of numbers by pressing the numbered buttons (0-9) in the same sequence (forward), or, in the second part of the test, in the reverse sequence (backward).
4. The Selective Attention Test (SAT) displays two squares located in the middle of the left and right sides of the screen. The subject is instructed to press the 3 key when a dot appears in the left

square and to press the 7 key when a dot appears in the right square. When dots appear outside of the squares the subject is to make no response.

5. The Serial Digit Learning (SDL) test sequentially presents a nine digit number on the screen and the subject is to reproduce the sequence.
6. The Tapping Test (TAP) instructs participants to press a button as many times as they can in a fixed period of time. Multiple trials are given for both the dominant and non-dominant hands.
7. The Continuous Performance Test (CPT) presents one of two stimuli in a random sequence, one of which the participant responds to by pressing a button, and for the other the participant withholds a response.
8. Progressive Ratio (PRT) requires participants to press a button on a ratio schedule in which the reinforcer (a smiling face) occurs progressively less frequently. The test measures motivation by recording when responses become infrequent or stop.
9. Reversal Learning (RLT) The participant is instructed to respond quickly on one button when they see any of three stimuli and on a different button when they see any of three different stimuli.

BARS tests employ much large stimuli for shaping instructions and for the test itself, as well as more clearly written instructions presented in large-size letters. Participants respond on a durable input device with large response buttons that is different from the typical 70+ button keyboard. The nine BARS performance tests were administered to the subjects in groups of 5-10 people each.

Data Analysis

The following approach was used in the analysis of the research questions posed in this investigation:

Do short-term biomarkers of exposure to pesticides (including insecticides and fungicides) differ in adolescents employed in agriculture compared to adult farmworkers controlling for type of agricultural work, hours worked, and reported hygiene practices?

Urinary metabolites were analyzed for all study participants. The distribution of urinary creatinine levels was evaluated and subjects with creatinine levels in the upper and lower 5% were removed from the analytical sample due to concerns regarding hydration state and underlying metabolic disorders. Standardization of the DAP metabolites to creatinine (nmol/g creatinine) was performed in the statistical analysis. The molar equivalent concentration of the DAPs were summed to create a summed DAP measure. Non-detects will be treated as zeroes in the summation of molar concentrations, and if the total DAP value is zero, it was assigned the value of 0.5 LOD for the DMDTP metabolite, the DAP with the lowest LOD.

Potential effect variables were determined from the interview questionnaire. The metabolite levels were examined among the farmworker sample according to hours worked in agriculture in the week prior to data collection (<30 or 30 or more), reported mixing/applying agricultural chemicals (yes/no), and hygiene index score (sum of 4 items on questionnaire: taking boots off, time interval before changing work clothes, time interval before showering after work; and washing hands before eating at work). The farmworker sample was stratified according to age (< 16, 16-18, 19-24, and 25 and older) to assess age related differences in pesticide metabolite levels.

A secondary research question that was addressed was the assessment of any differences in the biomarkers of pesticide exposure between the farmworker and non-farmworker samples, adjusted for age and gender. Urinary metabolites were log-transformed to improve symmetry and better approximate a normal distribution. A general linear model was used to examine how the hygiene index score and a subject's prior history of mixing/applying pesticides affects their urinary metabolite levels.

Is there evidence of a correlation between biomarkers of exposure to organophosphate pesticides and neurobehavioral performance in adolescent farmworkers and is that correlation similar to that observed among adult farmworkers?

The metabolites that were used for this analysis were the organophosphate metabolites because of the scientific evidence of the neurotoxic effect of organophosphate pesticides. The hypothesis that low-concentration pesticide exposures produce adverse neurobehavioral effects in adolescent farmworkers and this effect differs from that observed in adults, was tested by comparing the neurobehavioral performance of the adolescent farmworkers to the adult farmworkers and the controls.

A factor that had to be considered in the analysis of neurobehavioral performance is that, hypothetically, pesticide exposure can affect neurobehavioral performance both from an acute pharmacological effect at the time of testing (this would be demonstrated in the correlation between performance and the short-term markers of urinary pesticide metabolites) and a cumulative effect from low-dose, chronic exposure. Therefore it was important in these analyses to control for the total number of years of farm work experience.

We examined performance scores for the different groups of adults and youth using multiple regression models incorporating age, exposure group, gender, and other potential covariates such as the individual's familiarity with computers, and cumulative years of farmwork. Gender was added to the models if performance differed between the sexes or if linear trends for one or more of the factors was modified (through an interaction) by sex. Agricultural status was not used in the models because many of the controls participants were found to have spent at least 2 years previously working in agriculture. Participants with incomplete data on a neurobehavioral performance test were excluded from the analysis of that test.

Is there an association between exposure to agricultural pesticides and markers of DNA damage and oxidative stress in agricultural workers and does this association differ between adult and adolescent farmworkers controlling for work history, hygiene practices and lifestyle factors.

We formulated exposure groups in order to examine the association between exposure to agricultural pesticides and markers of DNA damage and oxidative stress. Questionnaire data were analyzed to identify individuals who reported that they are currently mixing/applying agricultural chemicals. We also reviewed the results of the pesticide metabolite data among the farmworkers, and assign subjects to exposure quartiles according to known handling exposure and mean levels of the urinary metabolites. We compared the DNA biomarker findings for this "pesticide handlers" group to an age-matched sample of farmworkers who do not report this work activity and do not have high urinary pesticide metabolites.

We also reviewed the results of the pesticide metabolite data and formulated among the farmworker sample quartiles according to the mean levels of the urinary metabolites. The markers of DNA damage were compared for the groups of farmworkers in the lower and upper quartiles for pesticide biomarkers.

The comet assay provides three parameters of cellular DNA damage for each comet: tail length (μm); tail fluorescence intensity (percent of DNA in tail) and tail moment (roughly the product of tail length and tail intensity). We calculated for each subject a mean score (on a continuous quantitative scale) for each of these parameters. Subjects' mean scores were combined to allow calculation of group means according to exposure and age. Group means (and variance) were compared and tested for significance using a general linear model.

Oxidative stress urinary markers were expressed as quantitated levels per kg body weight: nmol 8-oxodG/liter, nmol Tg/liter and μmol MDA/liter. Group means (and variance) were compared and tested for significance using a general linear model.

We evaluated as potential confounders several variables related to work and exposure: hours worked, reported mixing/applying, and hygiene index score. We also evaluated several factors with the potential to modify our marker measurements: gender, alcohol use, diet (consumption of red meat, caffeine, green tea, vegetables and fruits high in antioxidants/carotenoids), physical labor, and sunlight exposure (induces DNA repair). These data were collected through the interview questionnaire. Body mass index was calculated from height and weight data collected by interviewers.

RESULTS

We recruited a total of 407 participants in this study (125 controls and 282 farmworkers). The total agricultural group did not differ from the control groups, but the adolescent controls tended to somewhat younger than the adolescent farmworkers, while the adult controls were approximately 3 years older than the adult farmworkers. Among the adult agricultural workers and controls, more than two-thirds of the sample were male, however in the adolescent groups approximately half of the samples were female ($p < .001$). Eight of the adolescent farmworkers reported that they were working in the U.S. unaccompanied by their parents.

A large proportion of our farmworker sample spoke neither English nor Spanish as their primary language. In these subjects, their primary language was an indigenous language common to rural areas of Mexico and Central America. Approximately 12 % of our sample reported they were currently handling pesticides. Adults were more likely than adolescents to report currently handling pesticide ($p = .004$) or having handled pesticides in the past ($p < .0001$). Four of our controls were found to have self-reported currently handling pesticides. Two of the controls were in construction, one worked in landscaping and the fourth worked in a brake shop. Adults reported a mean of 9.35 years of agricultural work compared to adolescents who reported a mean of 2.62 years ($p < .0001$).

Table 1. Description of study participants.

	Farmworkers		Controls	
	Adolescents (n = 86)	Adults (n = 188)	Adolescents (n = 64)	Adults (n = 60)
Sample recruited 2004 2005	 42 44	 86 102	 25 39	 31 29
Mean Year of Age (sd)	15.7 (1.59) ****	28.1 (8.17)***	14.7 (1.46)	31.3 (9.13)
% Male	67.4	72.2	52.5	49.1
Primary Language n (%) Spanish English Other	 46 (53.5%) 0 (0) 40 (46.5%)	 93 (49.5%) 0 (0) 95 (50.5%)	 58 (95.2%) 1 (1.64%) 2 (3.28%)	 50 (94.3%) 3 (5.66%)
Years of Agricultural Work (sd)	2.62 (2.69)	9.35 (7.34)	0.6 (1.62)	3.31 (5.5)
Past Pesticide Handling n (%)	6 (7.06%)	53 (28.0%)*	7 (15.0%)	12 (20.3%)
Current Pesticide Handling n (%)	3 (3.53%)	22 (11.8%)**	0	4 (7.83%)

*p < .0001, ** p = .004, ***p < .05, ****p<.0005

The next tables shows the self-reported protection and hygiene practices of our subjects who were currently working in agriculture. The self-reported use of protection practices and hygiene were high with no significant differences between adolescents and adults. Adults who reported they currently were mixing or applying pesticides did not differ from other farmworkers on these reported practices.

Table 2. Self-reported protection and hygiene practices

Protection Practice or Hygiene	Adolescents (n = 86)	Adults (n = 188)
% Always Wash Hands Before Eating at Work	60.7	69.4
% Always Wash Hands Before Eating at Home	89.3	92.0
% Change clothes Immediately After Work	40.5	45.2
% Take off Work Boots Before Entering Home	77.4	73.7
% Showering Immediately after Work	21.4	18.8
% Always washing work clothes separately	91.6	87.6
Mean Protection Score (sd)	8.39 (1.64)	8.33 (1.93)

Specific Aim# 1: Do short-term biomarkers of exposure to pesticides (including insecticides and fungicides) differ in adolescents employed in agriculture compared to adult farmworkers controlling for type of agricultural work, hours worked, and reported hygiene practices?

We first analyzed this question with the subjects that were recruited in 2004. In this sample 186 subjects provided urine samples for analysis of biomarkers of pesticide exposure. We examined the levels of DMTP, the most prevalent organophosphate metabolite and also analyzed the samples for the presence of the metabolite of the fungicide Captan, which is used close to the harvest time of the berry crops.

Subjects with creatinine levels less than the 5th percentile (31.25mg/dl) or greater than the 95th percentile (227.5mg/dl) were removed. This led to an additional 20 subjects being removed (10 from each end) and 166 subjects for the final analysis. Non-detectable levels were replaced by one-half the minimum detectable level/limit (MDL) prior to analysis. The MDL was 2.2 ng/ml for the organophosphate metabolite DMTP, and 0.05 µg/ml for the Captan metabolite (THPI).

Levels of DMTP (ng/ml) among teens working in agriculture were shifted slightly higher compared to agricultural adults, though not by a significant amount (2-sided *p*-value = 0.07; Wilcoxon test). There were no differences in levels of THPI (2-sided *P-value* = 0.60; Wilcoxon test). Levels of DMTP were not significantly higher in all of the agricultural subjects combined relative to subjects not working in agriculture (1-sided *p*-value = 0.63; Wilcoxon test). Even after adjusting for age, levels of DMTP among agricultural subjects were not significantly higher than those found in non-agricultural subjects (1-sided *p*-value = 0.64).

Levels of THPI (µg/ml) were shifted significantly higher in the agricultural workers relative to the controls (1-sided *p*-value = 0.01; Wilcoxon test). However over 50% of both groups had non-detectable levels (0.025 µg/ml) of THPI. Specific tests of various percentiles (median, 60th, and 75th percentile) indicated that while medians did not differ in these two populations (1-sided *p*-value = 0.91), the 60th and 75th percentiles are both significantly higher in the agricultural population (60th percentile, 1-sided *p*-value = 0.01; 75th percentile, 1-sided *p*-value = 0.037).

Table 3. Pesticide Metabolite Levels, 2004 Sample

Sample Group	DMTP (ng/mL)			THPI (Captan metabolite)		
	Mean	Median	SD	Mean	Median	SD
Agriculture (n = 121)	12.0	1.1	38.0	0.14	0.025	0.15
Adults (n = 77)	8.3	1.1	20.0	0.15	0.025	0.18
Teens (n = 44)	20.0	3.2	56.0	0.11	0.073	0.10
Non Agriculture (n = 45)	14.0	1.1	32.0	0.078	0.025	0.10
Adults (n = 23)	9.3	1.1	21.0	0.06	0.025	0.08
Teens (n = 22)	20.0	3.9	40.0	0.10	0.025	0.12

Similar low exposures were found in the analysis of our 2005 study sample. Sums were computed for the methyl metabolites (DMP + DMTP + DMDTP) as well as the thiomethyl metabolites (DMTP + DMDTP). The 5th and 95th percentiles for creatinine levels were equal to 20 and 250 mg/dL, respectively. Subjects with creatinine levels less than 20 mg/dL or greater than 250 mg/dL were excluded from analysis due to hydration concerns, resulting in 164 samples available for analysis. Two additional subjects had to be removed from analysis due to missing data on agricultural status, resulting in a final analytical sample of 162 subjects of which 108 were working in agriculture at the time of the urine collection and 54 controls.

The mean age of the agricultural sample was 25 years (SD =10) and the non-agricultural sample had a mean age of 22 years (SD = 12). Seventy four percent of the agricultural worker sample was male; however the proportion of males among the non-agricultural samples was only 46%.

The levels of organophosphates in the urine samples were low. Only 11% of the agricultural samples and 5.6% of the controls had detectable levels of DMP. DEP and DETP were not detected in any samples. DMTP a very common metabolite of organophosphates was detectable in only 26% of the agricultural samples and 16% of the controls. Similarly, DMDTP was only detected in 15% of the agricultural samples and 11% of the

non-agricultural samples. The following table shows the mean and median values of the organophosphate metabolites in the agricultural and non-agricultural samples.

Table 4. Pesticide Metabolite Levels, 2005 Sample

Organophosphate Metabolite	Non-Agricultural (n = 54)		Agricultural (n = 108)	
	Mean (µmol/L)	Median (µmol/L)	Mean (µmol/L)	Median (µmol/L)
DMP	0.025	0.016	0.019	0.016
DEP	ND	ND	ND	ND
DMTP	0.066	0.0075	0.027	0.0075
DMDTP	0.016	0.005	0.012	0.005
DETP	ND	ND	ND	ND

ND = Non-detectable in all samples

Levels of all detectable organophosphate metabolites were observed to be *lower* in the agricultural samples strongly suggesting that there were no significant exposures to organophosphate pesticides during the time the subjects were studied. This was not surprising since organophosphates are not sprayed close to the time of berry harvesting.

Levels of THPI (µg/ml) in our 2005 sample were not significantly higher in agricultural workers compared to non-agricultural workers (1-sided p-value = 0.187; Wilcoxon test). The median among agricultural workers was observed to be 0.036 (mean = 0.065), while equaling 0.030 (mean = 0.056) for non-agricultural workers. Among the 118 agricultural workers for which age was known, 49 were teenagers (age <= 18) while 69 were adults (age >= 19). Levels of THPI did not significantly differ between teen and adult agricultural workers (2-sided p-value = 0.185; Wilcoxon test). The median level (µg/ml) among agricultural teens was found to be 0.04 (mean = 0.07) while equaling 0.03 (mean = 0.06) among agricultural adults.

Specific Aim # 2: Is there evidence of a correlation between biomarkers of exposure to organophosphate pesticides and neurobehavioral performance in adolescent farmworkers and is that correlation similar to that observed among adult farmworkers?

We have published the results of the analysis of neurobehavioral performance among the 2004 study participants. A total of 175 individuals completed the neurobehavioral test battery in the summer of 2004. While every attempt was made to recruit individuals with no agricultural experience our work history data revealed that 72% of the control adults and 37% of the control adolescents had some history of previously working in agriculture, although not currently doing so during the summer of 2004. Five controls had at least 8 years of agricultural experience with two reporting 10 years and one reporting 14 years. Approximately 20% of the sample (n = 38) reported that that they had mixed and/or applied pesticides in the past, and 17 participants had mixed or applied pesticides in the past month.

We determined to what extent farmworkers and controls were able to complete all components of the neurobehavioral test battery. The majority of participants completed all of the neurobehavioral tests; however, adult female participants working in agriculture had lower completion rates (75% of the neurobehavioral tests) compared to other groups ($t173 = 4.48$, $p < 0.001$) that had an average of 88% completion rate. A large percent of all participants was unable to complete the Reversal Learning test (approximately 68%). This was the last test presented in the lengthy battery and often participants did not have enough time to complete the test. The data from this test were excluded from the subsequent analyses.

The impact of age, years of education, gender and years working in agriculture was examined on each neurobehavioral measure. Table 5 presents the estimated slope (b-coefficient) showing the average change in each neurobehavioral measure per 5-year increase in the indicated predictor (age, years of education, years working in agriculture).

Table 5. Estimated slope (beta coefficient) showing the average increase or decrease in each neurobehavioral measure per 5-year increase in the indicated predictor (years working in agriculture, years of education, age); negative values for latency measures (Symbol-Digit, Reaction Time, Selective Attention) indicate improved performance. Measures that showed an interaction with gender are described separately for males (M) and females (F). One sided p-values are given in parentheses.

Neurobehavioral Measure	Years in Agriculture	Years of Education	Age	Notes
Digit Span				
Forward		0.49 (<0.01)		
Reverse		0.34 (0.01)		
Finger Tapping				
Preferred			-2.4 (0.99)	Gender[1]
Non-preferred			-1.4 (0.97)	Gender[2]
Alternating		4.70 (0.01)		Gender[3]
Symbol-Digit				
Latency	M: -10 (0.57) F: 480 (<0.01)	-300 (<0.01)	155 (0.99)	
Match-Sample				
Score	-0.61 (0.03)	0.75 (0.05)		
Reaction Time				
Latency	M: -12 (0.94) F: 32 (<0.01)	-21 (0.02)		
Selective Attention				
Trials		-16 (0.02)	F: -12 (-.96) M: -5.9 (0.12)	AGxAge[4]
Latency			F: 19 (0.99)	
Serial Digit Learn				
Score		2.6 (< 0.01)	-0.7 (0.98)	
Cont. Performance		3.8 (0.02)		
% Hits		-6.5 (<0.01)		
% False Alarms		-3.7 (0.02)		
% Omissions		0.6 (< 0.01)		AGxAge[5]
D-prime				
Progressive Ratio				
Number Taps				Gender[6]

(1) Significant overall effect due to sex (p<0.01); females averaged 17.4 fewer taps than males.
(2) Significant overall effect due to sex (p<0.01); females averaged 11.7 fewer taps than males.
(3) Significant overall effect due to sex (p<0.01); females averaged 13.2 fewer taps than males.
(4) Males showed an interaction between age and years in agriculture (p=0.01); performance worsened for males as both age and years in agriculture increase together.
(5) Significant interaction between age and years in agriculture (p=0.04); for older participants (>=35), scores increased as both age and years in agriculture increase together.
(6) Significant overall effect due to sex (p<0.01); females averaged 87 points lower than males.

In testing our study hypothesis we found that adolescents did not have poorer performance on the neurobehavioral test battery. We found higher performance among young workers, as the age of the participants increased the performance on the Finger Tapping (preferred and non-preferred trials), Symbol-Digit, and Serial Digit Learning tests decreased. For example, performance on Finger Tapping (preferred hand) decreases an average of 2.4 taps for each 5-year increase in age. In addition, an interaction between age and gender was found for the Selective Attention Test (number of trials, latency). The older the female participants, the more performance on the Selective Attention measures decreased. The only measure that had improved performance for the adults was the d-prime measure of the Continuous Performance test (a measure of

attentiveness, how well a participant distinguishes between targets and non-targets). In general, older subjects tended to have slightly higher scores on the Continuous Performance test than did younger subjects with similar years spent working in agriculture. For those older subjects, scores appeared to increase with increasing years spent working in agriculture; for younger subjects, average scores tended to decrease.

Education was found to strongly influence performance on the tests. Years of education in the participant's country of origin was found to have a significant main effect on Digit Span (forward and reverse), Finger Tapping (alternating trials), Symbol-Digit, Reaction Time, Selective Attention (latency), Serial Digit Learning, Match-to-Sample (score), and Continuous Performance. In each case as years of education increased performance on these measures improved.

A significant main effect of gender was found on the Finger Tapping (preferred, non-preferred, and alternating trials) and Progressive Ratio Tests with females performing worse than males on these two tests.

A significant main effect of years working in agriculture was found for Match-to-Sample (score), as years spent working in agriculture increased performance decreased. An interaction between age and years working in agriculture was found for Continuous Performance (d-prime), older participants tended to have slightly better scores than younger participants with similar years spent working in agriculture. Gender was also found to interact significantly with years working in agriculture on the Symbol-Digit, Reaction Time, and (for male participants) Selective Attention tests. For females, as years working in agriculture increased, performance on the Symbol-Digit and Reaction Time measures decreased; this effect was not significant for males. For males there was no effect on Symbol-Digit or Reaction Time. However, there was a compound effect of age and years working in agriculture for the males (linear _ linear interaction). As both age and years of working in agriculture increased in males, performance on the Selective Attention measures decreased. For females, as age increased, performance on the Selective Attention measures decreased.

Neurobehavioral performance was examined in men (n = 108) to determine whether differences existed among three groups: those without any experience mixing/applying pesticides (68%; n = 74), those with any prior experience of mixing/applying pesticides (31%; n = 34) and a subset of the previous group of men who had mixed/applied pesticides in the month prior to testing (15%; n = 16). Multiple linear regression was used to control for differences due to age, years of education, and years spent working in agriculture. The expectation was that current or past mixing/applying pesticide activity would be associated with lower neurobehavioral performance; consequently, one-sided p-values were used in comparing groups with some prior experience mixing/applying pesticides against the baseline group of non-mixer/applicators. Any experience of mixing/applying pesticides was found to significantly decrease performance on four neurobehavioral measures. Scores on Digit Span forward and Digit Span reverse were significantly lower for men who had handled pesticides (0.51 points lower for forward, p = 0.02 and 0.52 points lower for reverse, p = 0.02). Match-to-Sample scores were also lower (2.04 points) for men who reported handling pesticides in the past compared to men who had never reported handling pesticides (p = 0.02). The percentage of hits on the Continuous Performance test also showed a decrease for men who handled pesticides (6.4 percentage points, p = 0.047). Although not significant, performance was also decreased on Serial Digit Learning (2.02 points lower among men who had handled pesticides; p = 0.09) and Symbol-Digit (average latency 135 ms greater, p = 0.21). The Progressive Ratio test showed improved performance for men who had handled pesticides in the past (41.7 points higher).When the subset of participants who had recent experience mixing/applying pesticides was compared to the participants who had no experience handling pesticides, three neurobehavioral measures showed decreased performance. Men who reported mixing/applying pesticides in the past month had an average Match-to-Sample score 2.68 points lower than participants with no experience handling pesticides (p = 0.015). The percentage of hits and d-prime score for the Continuous Performance test also showed decreased performance, 15.8 percentage points on percent hits and 0.79 points lower on d-prime score, for men mixing/applying pesticides in the past month compared to men with no pesticide handling (p = 0.001 and p = 0.012, respectively). The Progressive Ratio test showed that men who had recent experience mixing/applying pesticides had improved performance (25.8 more taps) compared to men with no experience handling pesticides (one-sided p-value = 0.85).

Specific Aim # 3: Is there an association between exposure to agricultural pesticides and markers of DNA damage and oxidative stress in agricultural workers and does this association differ between adult and adolescent farmworkers controlling for work history, hygiene practices and lifestyle factors?
This question was first analyzed for the 2004 sample. One-hundred thirty-four (134) Latino farmworkers who were working in agriculture at the time of recruitment and 55 Latino individuals who

reported not currently working in agriculture participated in 2004 and provided biological samples (urine or buccal cells) for analyses. Table 6 shows descriptive statistics for factors that could be confounding variables on the measures of DNA damage and oxidative stress. Means were analyzed using t-tests while proportions were analyzed using chi-square tests. Two dietary variables (carotenoid and antioxidant intake) were analyzed using a Wilcoxon rank-sum test.

Table 6. Frequency of Factors Associated with Oxidative Stress in Study Sample

	Agricultural Workers (n=134)	Controls (n=55)	p-value
Mean Years of Age (sd)	23.3 (8.6)	23.1 (10.2)	0.87
BMI, mean (sd)	25.4 (4.5)	26.1 (6.5)	0.52
Carotenoid intake 25^{th}, 50^{th}, 75^{th} %tile	5, 6, 7	4, 6, 7	0.18
Antioxidant intake 25^{th}, 50^{th}, 75^{th} percentile	10, 11, 13	8, 11, 13	0.19
% Smoker	9.0	10.1	0.68
% Alcohol	21.6	25.5	0.57
Exercise (> 5 hr/wk)	11.2	21.8	0.02
Recent illness (%)	35.1	36.4	0.87

One hundred thirty-nine subjects (102 Agricultural Workers, 37 Controls) submitted samples in 2004 for comet analysis. Eight subjects had fewer than 10 cells for analysis and were excluded, leaving 131 subjects (98 Agricultural workers, 33 non-Agricultural workers) with sufficient data (>=10 cells/assay). Of these 131 subjects, 68% had exactly 50 cells/assay, while 21% had assays based on 10–30 cells.

No significant difference was found between agricultural workers and controls with respect to tail length (1-sided p-value = 0.17); however, the mean tail intensity was significantly greater for agricultural workers compared to controls (1-sided p-value < 0.001). On average, the mean tail intensity was 10.9 units greater for agricultural workers (95% CI 6–16 units greater). Tail moment was also significantly greater for agricultural workers compared to non-agricultural workers (1-sided p-value < 0.001). The median tail moment for agricultural subjects is estimated to be 43% greater than for non-Ag subjects (95% CI: 17–75% greater). No comet parameter was significantly associated with years spent working in agriculture (2-sided p-values = 0.40, 0.93, 0.46 for tail length, tail intensity, and tail moment, respectively). These same comet parameters showed no significant association with age (2-sided p-values = 0.37, 0.10 and 0.17 for tail length, tail intensity, and tail moment, respectively) or sex (2-sided p-values = 0.93, 0.97 and 0.84 for tail length, tail intensity, and tail moment, respectively).

Among the 98 agricultural workers having sufficient number of cells for comet assay, 36 were teenage workers and 62 were adults. The parameters tail length, tail intensity and tail moment were compared between the two groups using a mixed-effect model with tail length and tail moment being log-transformed prior to analysis. Median tail length and tail moment did not significantly differ between teen and adult agricultural workers (2-sided p-values = 0.44 and 0.68 for length and moment, respectively). There was also no significant difference between the two groups with respect to mean tail length (2-sided p-value = 0.84). The following table shows the estimated response for each group together with 95% confidence intervals.

Table 7. Comparison of comet values in adult and adolescent farmworkers

	Teen (n=36)	Adult (n=62)
Tail length, median (CI)	0.047 (0.040, 0.055)	0.043 (0.038, 0.049)
Tail moment, median (CI)	0.01 (0.0088, 0.012)	0.01 (0.0088, 0.011)
Tail intensity, median (CI)	38 (34, 42)	38 (34, 41)

One hundred thirty-six subjects (100 Agricultural, 36 controls) submitted both a urine sample and a buccal sample for comet analysis. Of these, 118 samples (90 agricultural workers, 20 controls) simultaneously satisfied the restrictions placed on creatinine (>= 31.25 mg/dl, <= 227.5 mg/dl) levels and on the number of cells needed for reliable Comet analysis (>=10). The partial correlation was computed between each of the three aforementioned comet parameters and levels of DMTP, the Captan metabolite (THPI), and the molar concentration of the summed methyl metabolites while controlling for creatinine concentration, cigarette and alcohol consumption. The analysis was restricted to the 90 agricultural workers.

The following table shows the partial correlations among n=90 agricultural workers between comet parameters and DMTP, THPI, and combined concentration of methyl organophosphate metabolites. The analysis adjusted for creatinine concentration, cigarette consumption, and alcohol consumption. The one-sided p-value is shown in parentheses. These results did not provide any statistically significant correlation between the degree of DNA damage and the levels of urinary pesticide metabolites.

Table 8. Partial Correlations between Comet parameters and urinary metabolites.

	DMTP	THPI	Methyl sum
Tail length	0.11 (0.17)	0.10 (0.17)	0.12 (0.13)
Tail intensity	0.14 (0.09)	0.09 (0.21)	0.15 (0.08)
Tail moment	0.16 (0.08)	0.15 (0.08)	0.17 (0.06)

Factors that might have confounded the observed differences in the comet results seen between the agricultural and nonagricultural groups were examined. Alcohol and cigarette use were dichotomized according to whether use was great than zero. Thirty-three subjects reported alcohol use and 9 subjects reported some tobacco use. Vegetable carotenoids and antioxidant consumption were treated as continuous variables. BMI was log-transformed prior to inclusion in the model. These five variables (plus the interaction between alcohol and cigarette use) were not significantly associated with average tail length ($F(6,124) = 1.92$, $p=0.08$), tail intensity ($F(6,124)=0.98$, $p=0.44$), or tail moment ($F(6,124)=1.84$, $p=0.10$).

We have also examined 8-oxodG, a marker of oxidative stress. A linear model with creatinine treated as a continuous covariate was used to estimate the difference in levels of log-transformed 8-oxodG. The reported difference, and its significance, was evaluated (estimated) for a subject with 130mg/dl creatinine. Subjects that worked in agriculture did not have significantly higher levels of 8-oxodG relative to those individuals not working in agriculture (1-sided p-value = 0.51). Levels of 8-oxodG were not significantly different between adults and adolescents working in agriculture (2-sided p-value = 0.37). We found no correlation between a subject's 8-oxodG level and their time (years) spent working in agriculture was not significant ($r_s = 0.063$, 1- sided p-value = 0.21; Spearman's correlation coefficient).

The following year (2005) we attempted to do the analysis of the comet analyses on fresh buccal samples to circumvent the methodological problems that we had encountered the previous year with the effects of cryopreservation on the subsequent viability of the leukocytes in the buccal samples. After removing samples with less than 10 viable cells we had 129 samples (69 frozen and 85 fresh). Analyses were performed separately for fresh and frozen samples. For each of these groups, a mixed-effect model was fitted to the particular comet parameter while treating AG status as a fixed factor and subject as a random effect. Tail moment and tail length were transformed prior to analysis (square-root or fourth-root) to improve symmetry; consequently, inferential statements concern changes in the median response. Head intensity was not transformed making inferential statements apply to the mean response. P-values are two sided.

Within the frozen samples, there were 56 from agricultural workers and 13 from controls. In the 69 samples of frozen buccal cells, we found that the median tail moment, tail length or head intensity did not significantly differ between agricultural subjects and controls G ($t67 = -0.19, p = 0.85$; mixed-effect model) ($t67 = -0.75, p = 0.45$; mixed-effect model), ($t67 = -0.12$, $p = 0.90$; mixed-effect model) respectively. However we were able to observe differences in the subjects for whom we analyzed their cells without freezing. We found that the median tail moment was significantly greater in agricultural workers relative to controls ($t83 = 3.61$, $p < 0.001$;mixed-effect model). In the control group, the median tail moment was estimated to be 0.77 (95% CI: 0.65–0.91), while being 1.1 (95% CI: 0.98–1.2) in the agricultural group. We also found that the median tail length was significantly greater in agricultural workers relative to controls ($t83 = 3.81$, $p < 0.001$; mixed-effect model). In the control group, the median was estimated to be 4.1 (95% CI: 3.7–4.4), while it was estimated to be 4.9 (95% CI: 4.7–5.2) in the agricultural group. Likewise the mean head intensity was significantly lower in the agricultural workers relative to the control group ($t83 = -3.57$, $p < 0.001$). The mean head intensity was estimated to be 7.7 units lower in the agricultural group compared to the control group (95% CI: 3.4–12 units lower). Average head intensity for the control group was estimated to be 66 units (95% CI: 61–68), while being 57 units (95% CI: 54–59) in the agricultural group.

To assess the effect of cryopreservation on the cell detail we analyzed the samples of 26 individuals for whom we analyzed their samples both pre-and post-cryopreservation. The majority (15/26) had at least 50 cells for analysis from *both* conditions (fresh and frozen). Fresh and frozen samples are examined for both shifts in and changes in dispersion (i.e., does within-subject variation differ between fresh and frozen samples?). Shifts in mean/median between fresh and frozen samples were assessed with a mixed-effect model containing a factor for type (fresh/frozen; a fixed effect) and subject (the 26 individuals sampled; a random effect).Testing whether within subject variation differed by sample type was done using the median-modified version of Levene's test (81,82). The test for variation was applied to the comet parameters with, and without, transformation. In all cases conclusions were the same regardless of whether data had been transformed, so the test statistic and *p*-value are reported for the untransformed response. Fresh and frozen samples showed no significant shift in median tail length ($t25 = 1.13$, $p = 0.27$) but did appear to have differing amounts of within-subject variation ($F1,2387 = 43.2$, $p < 0.001$). Fresh and frozen samples also showed no significant shift in median tail moment ($t25 = 1.37$, $p = 0.18$), but did appear to have differing amounts of within-subject variation ($F1,2387 = 4.05$, $p = 0.044$). The average head intensity for frozen samples was approximately 6 units lower (95% CI: 0.8–12 units lower) than for fresh samples ($t25 = -2.36$, $p = 0.026$). Freezing samples had no significant effect on within-subject variation ($F1,2387 = 1.07$, $p = 0.302$).

DISCUSSION

Adolescent Vulnerability

This work was initiated in response to the large number of youth in the U.S. today hired as part of the agricultural labor force on a migrant or seasonal basis. As noted by others, we found a significant proportion of the adolescents in our sample of farmworkers working away from their parents as unaccompanied minors. In a survey of migrant adolescent farmworkers in Oregon, we previously found that 64.7% of the adolescents living in migrant labor camps were traveling and working in the U.S. unaccompanied by their parents(2). In this sample we found that *** of our adolescent workers fit that category.

The adolescent farmworkers in our sample were very similar in every aspect to their adult farmworkers. Though they were less likely to report handling pesticides, their work activities and use of protection were very similar to adults. There performance on the neurobehavioral tests did not indicate that they might be having neurological effects from exposures to low levels of pesticides in the workplace. However, we did find that cumulative years of agricultural work were associated with poorer neurobehavioral performance controlling for age at the time of the test. Therefore individuals beginning agricultural work at an early age and sustaining this employment may be more likely to develop effects on neurobehavioral performance. Longitudinal studies of the farmworker workforce would be needed to more clearly demonstrative these cumulative effect. However, the seasonal and migratory nature of agricultural work makes the design of such investigations extremely difficult.

Neurobehavioral Results

Age, school experience, gender, and years working in agriculture all impacted performance on the neurobehavioral tests. Pesticide handling was also associated with performance on the neurobehavioral tests. Age had an impact on the Finger Tapping, Symbol-Digit, Selective Attention and the Continuous Performance

(d-prime) tests. With the exception of Continuous Performance, older participants performed worse than younger participants. Years of education had a significant impact on performance on eight out of nine neurobehavioral tests. As years of education increased, performance on the neurobehavioral tests improved. Gender also had an impact on performance. On the motor tests, Finger Tapping and Progressive Ratio, females performed worse than males. This is consistent with Anger et al. (83), who also found an effect of gender on a tapping measure in the same direction. These findings suggest that years working in agriculture also impacted performance. More years working in agriculture was associated with worse performance on the Match-to-Sample, Symbol-Digit, Reaction Time, and Selective Attention tests.

There also appears to be an interaction between years working in agriculture, age and gender on a number of measures. Similar gender effects were reported in an earlier study conducted Rothlein et al. (84). While gender differences have been noted on specific neurobehavioral tests, these results and earlier findings suggest that there may be a differential impact from agricultural work as well. Other studies have also found lower performance on neurobehavioral tests associated with increased years working in agriculture (85,86). Participants chronically exposed to pesticides for more than 10 years had lower performance on measures of perception and visuospatial processing (86). This study also showed no correlation between plasma cholinesterase, a measure of recent exposure, and cognitive deficits. Kamel et al. also found that the greatest decrease in cognitive and psychomotor functions was observed after 10 or more years of work (85).

Handling pesticides also impacted neurobehavioral performance. In our 2004 sample, 34 participants report mixing and applying pesticides. More males than females (34 versus 4) and more adults than adolescents (30 versus 8) reported handling pesticides. The years working in agriculture were very similar for the adult male participants who never handled pesticides (10.6 years and 2.1 years for the agricultural and non-agricultural groups) compared to the adult male participants who report mixing/applying pesticides (9.4 years and 3.0 years for the agricultural and non-agricultural groups). However, the male adolescent participants who never handled pesticides had fewer years working in agriculture (2.8 years and 1.5 years for the agricultural and control groups) compared to the male adolescents who report mixing/applying pesticides (8.0 years and 5.5 years for the agricultural and control groups). Performance deficits associated with pesticide handling were found on the Digit Span, Match-to-Sample, and Continuous Performance Tests. Interactions found between neurobehavioral performance and demographic variables such as age, education, and gender have been known to impact performance on neurobehavioral tests (83).

Several neurobehavioral measures were significantly affected by the gender of the participant. Previous studies of neurobehavioral performance in farmworkers have generally assumed that observed deficits are a result of pesticide exposure (85) and significant gender effects in humans have not been reported. Rothlein et al. reported gender differences on Finger Tapping, Serial Digit Learning and an overall summary index of neurobehavioral performance in Oregon farmworkers (84). Furthermore, several findings examining organophosphate exposure in rats have demonstrated differential effects of gender (87, 88, and 89). Further research is warranted to examine the impact of gender.

These findings do not provide evidence that adolescents working in agriculture are more likely to perform more poorly on these tests than their adult counterparts. However, the results are limited in that no exposure variables are available other than years of working in agriculture and self-reported pesticide handling activity. At the time that the subjects were tested, their exposures to organophosphate pesticides were low according to their urinary metabolite levels. We did not have information on the types and amounts of pesticide exposures the subjects might have had in the weeks and months prior to their testing. The results of four tests (Match-to-Sample, Selective Attention, Symbol-Digit, and Reaction Time) add to the increasing evidence that neurological impairment may be associated with increased years working in agriculture. Furthermore, the deficits found in the participants who reported handling pesticides compared to those with no experience indicate the potential impact of pesticide exposure. Time and exposure levels need to be examined to determine the dose–effect relationship. Longitudinal studies are needed to document if earlier onset of agricultural work results in increased deficits as a cohort ages.

Comet Results

In the last decade, the comet assay has been used increasingly in studies of workers potentially exposed to genotoxic substances in the workplace or environment. Faust et al. reviewed the use of the comet assay in 30 occupational studies (90). The objective of that review was to determine whether human lymphocytes are relevant target cells for monitoring purposes or if non-blood cells, such as exfoliated buccal cells, could provide a more precise prediction of health effects. Three agricultural studies were reviewed in this panel, all

conducted on pesticide plant workers and each showing a significant relationship between comet results and occupational exposure.

Cigarette smoking is assumed to be a potential confounder of studies of pesticide exposure and DNA damage due to the large number of genotoxic substances in tobacco smoke. We did not find a significant relationship in our results. This is particularly surprising since Faust suggests that exfoliated oral or nasal cells might be more susceptible to damage given the direct contact of cells from these locations and tobacco agents. The amount of smoking could be a major determinant of DNA damage. Likewise, we did not find a significant relationship between age of the subject and levels of DNA damage. As Faust has suggested, while it seems reasonable to speculate that DNA damage and comet parameters may increase in older subjects as compared to younger ones, most studies do not support this assumption. Our sample also consisted of a large proportion of younger workers who could be having rapid growth, thereby increasing the risk of DNA damage. However we found very similar levels of DNA damage in both our adult and adolescent farmworkers.

A strength of our study was the large number of subjects and the ability to control for confounding variables such as physical activity, diet, and ethnicity. These potential confounders including dietary antioxidants, exercise, sunlight, and air pollution are virtually never important determinants in cross-sectional studies (61, 91). Nonetheless, the major different between our agricultural workers and the controls in this study could be sunlight exposure related to agricultural work. Agricultural workers routinely wear long sleeve shirts, long pants and caps, however specific sun exposure was not assessed in this study. Future studies will investigate the relationship between DNA damage in occupational groups with comparable work outside, but with varying levels of pesticide exposure.

Minimally invasive methods of biomonitoring are needed in epidemiological studies of farmworker populations. The results of this study using buccal cell comet assays demonstrate the feasibility for use with large population studies and the sensitivity to detect differences in exposures to pesticides. We did however encounter significant methodological challenges in the storage/analysis of the buccal samples. Our previous studies have focused on DNA damage as detected in lymphocytes in venous blood (92). The extraction of lymphocytes and cryofreezing until analysis is a procedure that has been well documented in the literature. The preparation of buccal cell samples is much more critical. We found that using the same percent DSMO freezing preservative (10%) that preserves lymphocytes well, was too strong for the leukocytes that are analyzed in buccal samples. After cryopreserving the 2004 samples we found a large proportion of the cells did not survive the procedure. In the 2005 samples we attempted to do the comet assays within 48 hours of collection and to avoid cryopreserving. In the 2005 samples we have a proportion of the samples in which we have both cryopreserved and fresh cell analyses. We will compare the differences observed between our agricultural and non-agricultural groups to determine the extent in which cryopreserving affects the overall magnitude of differences observed between the agricultural and nonagricultural groups.

Additional Analyses and Manuscripts in Preparation

We have had one publication to date on the results of this study:

Rohlman DS. Lasarev M. Anger WK. Scherer J. Stupfel J. McCauley L. Neurobehavioral performance of adult and adolescent agricultural workers. *Neurotoxicology*. 28(2):374-80, 2007 Mar.

We currently have another manuscript in publication, "Pesticide Exposure and DNA Damage in Immigrant Farmworkers" that we plan to submit to the *Journal of Occupational and Environmental Medicine*. Analyses will continue on the database. Analysis plans include:

1. Further explore the relationship of cumulative years of agricultural work and neurobehavioral performance on entire sample. Our publication only includes subjects recruited in the first recruitment phase of the study.
2. Explore the hypothesis that gender could influence the health effects associated with pesticide exposure. We currently have a proposal under review exploring this hypothesis in a new sample of farmworkers. The completion of neurobehavioral outcomes in the entire sample supports this proposed work.
3. Examine the relationship between DNA damage as measured by the comet assay and neurobehavioral performance to see if the individuals with higher DNA damage show more impairment on the neurobehavioral tests.

4. To examine the relationship of DNA damage to cumulative years of agricultural work and a positive history of handling pesticides.
5. We are also developing a methodological paper on the comparison of the fresh and frozen buccal cells and the effect of those differences on observed effects of DNA damage associated with pesticide exposure.
6. We have decided to examine levels of isoprostanes in the urine samples obtained in this study. An individual's isoprostane level is now widely recognized as the most reliable biomarker for systemic oxidative stress.The isoprostanes are prostaglandin-like compounds formed *in vivo* from the free radical-catalyzed peroxidation of essential fatty acids and are accurate markers of lipid peroxidation in both animal and human models of oxidative stress.

CONCLUSIONS

In summary we found indications of very low pesticide exposures among the farmworkers in our study, and no significant differences in either organophosphate or fungicide metabolite levels between adolescents and adults. Surprisingly, even with these low exposures we found that farmworkers performed poorer than non-agricultural participants, but adolescent farmworkers did not have poorer performance than their adult counterparts. Performance on several tests decreased as years spent working in agriculture increased and any experience of mixing/applying pesticides was found to significantly decrease performance on four neurobehavioral measures (Digit Span forward, Digit Span backward, Match-to-Sample, and the Continuous Performance test). When the subset of participants who had recent experience mixing/applying pesticides was compared to the participants who had no experience handling pesticides, three neurobehavioral measures showed decreased performance.

On the comet assays for DNA damage we found that the mean tail intensity was significantly greater for agricultural workers compared to controls and tail moment was also significantly greater for agricultural workers compared to non-agricultural workers. No comet parameter was significantly associated with years spent working in agriculture and comet parameters were not significantly associated with urinary pesticide metabolites.The findings of significantly increased indicators of DNA damage among the farmworker participants is also of concern given the postulated relationship between DNA damage and subsequent development of a number of chronic disease and cancer. However we did not find any indication that adolescent farmworkers had more DNA damage than their adult coworkers. Median tail length and tail moment did not significantly differ between teen and adult agricultural workers. Farmworkers did not have significantly higher levels of the DNA adduct 8-oxodG relative to those individuals not working in agriculture, nor were levels higher in adolescents compared to adults.

The results of this study provide evidence that adolescents do not appear to have specific developmental susceptibility to pesticide exposures as measured by neurobehavioral performance and DNA damage. However this study adds to a growing body of evidence that chronic pesticide exposure in farmworkers is associated with effects on neurobehavioral performance. The sources and types of exposures to pesticides in populations who do not mix or handle pesticides needs further attention. Educational programs are needed to communication the results of this work and similar studies. The evidence from this study adds to a growing body of studies on the potential utility of biomonitoring DNA damage and oxidative stress among working populations as an indicator of potential health problems.

We have demonstrated the ability to access a large number of immigrant farmworkers for a scientific investigation on health effects associated with pesticide exposures and the results provide some reassurance of the safety of farmwork for adolescents, but the participants in this study were exposed to very low levels of pesticides, which might not pertain to all types of work experienced by this seasonal and migrant workforce

Program Director/Principal Investigator (Last, First, Middle): McCauley, Linda A.

Inclusion Enrollment Report

This report format should NOT be used for data collection from study participants.

Study Title: Biomarkers of Pesticide Toxicity Among Teen Farmworkers

Total Enrollment: 409 **Protocol Number:** 801372

Grant Number: R01 OH008057

PART A. TOTAL ENROLLMENT REPORT: Number of Subjects Enrolled to Date (Cumulative) by Ethnicity and Race

Ethnic Category	Sex/Gender: Females	Males	Unknown or Not Reported	Total
Hispanic or Latino	145	264		409 **
Not Hispanic or Latino				
Unknown (individuals not reporting ethnicity)				
Ethnic Category: Total of All Subjects*	145	264		409 *
Racial Categories				
American Indian/Alaska Native				
Asian				
Native Hawaiian or Other Pacific Islander				
Black or African American				
White				
More Than One Race				
Unknown or Not Reported	145	264		409
Racial Categories: Total of All Subjects*	145	264		409 *

PART B. HISPANIC ENROLLMENT REPORT: Number of Hispanics or Latinos Enrolled to Date (Cumulative)

Racial Categories	Females	Males	Unknown or Not Reported	Total
American Indian or Alaska Native				
Asian				
Native Hawaiian or Other Pacific Islander				
Black or African American				
White				
More Than One Race				
Unknown or Not Reported	145	264		409
Racial Categories: Total of Hispanics or Latinos**	145	264		409 **

* These totals must agree.
** These totals must agree.

Inclusion of Children

A major focus of this research was the examine the pesticide exposures among adolescent farmworkers, therefore approximately half of our study sample were individuals ages 18 and younger.

Materials available for other investigators

Electronic data from study questionnaires are available to other investigators and may be accessed by contacting the PI of the study.

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