UNIVERSITY OF CAPE TOWN

GENETIC POLYMORPHISMS AND ORGANOPHOSPHATE NEUROTOXICITY AMONGST EMERGING FARMERS IN THE WESTERN CAPE

TRACY GLASS

PART 0: PREAMBLE

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STUDENT NUMBER: GLSTRA003

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DECLARATION

I, Tracy Glass (GLSTRA003), hereby declare that the work in this mini dissertation is based on my original work (except where acknowledgements indicate otherwise) and has not, in whole or in part, been submitted towards another degree at this or any other university.

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Thesis abstract

BACKGROUND: Long-term exposure to organophosphates (OPs) can cause chronic neurotoxic effects which may be modulated by genetic polymorphisms of xenobiotic metabolising enzymes (XMEs). No previous study investigated XME modulation of neurotoxicity outcomes.

OBJECTIVES: To investigate whether XMEs polymorphisms modulate OP neurotoxicity among emerging farmers.

METHODS: A cross-sectional study of 301 emerging farmers was conducted in the rural Western Cape of South Africa. Neurotoxicity testing included the World Health Organisation Core Test Battery (digit span forward and backward) and vibration sensitivity testing. Questionnaire items included demographic data, potential confounders and work history of pesticide exposures. Blood samples were analysed for genetic polymorphisms of the following XMEs; glutathione S-transferases (GST), N-acetyltransferases (NAT) and Paraoxonase (PON1).

RESULTS: Median age was 39 (30-48) and most had 9 years of education or less (65.5%). 54% of the participants were OP pesticide applicators. There was a low prevalence of the GST null genotype (GSTT-1% and GSTM-16%) and the GA and GG genotype for NAT (10%). Modulation of OP exposure and neurotoxic outcome relationships by NAT, PON1 at position 192 and GST was indicated in multivariate analysis. The strongest evidence of modification was by NAT on the relationship between pesticide poisoning and impaired vibration sense. Poisoned individuals with the GG genotype were more likely to suffer from impaired vibration sense compared to GA and AA genotypes.

CONCLUSION: Genetic polymorphisms of NAT, PON1 (at position 192) and GSTM may modify the relationship between OP exposure and neurotoxicity. Larger longitudinal studies are required to determine whether preventive strategies can be developed to improve health amongst the identified vulnerable groups.

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PART A: Protocol

1. Literature Review

1.1. Introduction

Globally, organophosphates (OPs) are currently among the most widely used class of pesticides (Costa et al. 2005; Mackness et al. 1997; Singh et al. 2011a). OPs were previously used as nerve agents in chemical warfare and for crop protection in agriculture. OP pesticide-related chemical derivatives are also used as pharmaceutical agents for the treatment of glaucoma and schistosomiasis and for mosquito control in the prevention of transmission of malaria (Costa et al. 2003a).

Exposure to OP pesticides is associated with negative effects on the central nervous system (CNS). This is because OPs inhibit acetylcholinesterase (AChE), an enzyme that inactivates the neurotransmitter, acetylcholine. The inhibition of AChE results in the accumulation of acetylcholine which causes the over stimulation of postsynaptic cholinergic receptors and disrupts the transmission of neuron impulses at nerve endings (Alavanja et al. 2004; Costa et al. 2003b). Neurotoxic effects associated with OP exposure in humans vary with the duration and the quantity of the chemical that the individual is exposed to. Acute high dose OP exposure is associated with dizziness, increased salivation, nausea, vomiting and in more severe cases, respiratory difficulty and seizures. Long-term low-dose exposure to OPs can cause generalised weakness, tremors and a decrease in cognitive functions, such as impairment in memory and concentration. The harmful effects are less clear for low level, long term OP exposure, largely due to the difficulties associated with exposure measurement (Costa et al. 2005).

In addition to the acute symptoms described earlier, OP exposure has been linked to a wide range of adverse health outcomes including neurodegenerative diseases such as Parkinson's disease and Alzheimer's disease (Baldi et al. 2003; Freire and Koifman 2012; Le Couteur et al. 1999), cancers such as Non-Hodgkin’s lymphoma, leukemia and multiple myeloma (Mills and Zahm 2001), respiratory problems and asthma (Faria et al. 2005; Hoppin et al. 2008; Hoppin et al. 2009) and depression and anxiety (Mackenzie Ross et al. 2010). Previous studies have also found an association between prenatal maternal OP pesticide exposure and neurodevelopmental deficits in early childhood, in addition to low birth weight and birth length (Bouchard et al. 2011; Engel et al. 2007; Eskenazi et al. 2007; Grandjean et al. 2006; Whyatt et al. 2004; Young et al. 2005).

1.2. Neurotoxic effects due to long-term organophosphate pesticide exposure

1.2.1. OP pesticides and neurobehavioural performance

A number of epidemiological studies have investigated the effects of OP pesticides on neurobehavioural performance. The tests for neurobehavioural performance used in these studies include the World Health Organization Neurobehavioral Core Test Battery (WHO NCTB), the Q16 questionnaire and the Behavioural Assessment and Research System (BARS). A few studies found that OP exposure decreased neurobehavioural performance in adults including workers who were exposed to OPs through sheep dipping (Stephens et al. 1995), workers in greenhouses (Bazylewicz-Walczak et al. 1999), applicators and non-applicators on crop farms (Rohlman et al. 2007; Kamel et al. 2003).

However, some studies found a positive association between OP exposure and neurobehavioural performance while others found no association (Holtman 2013; London et al. 1997; Major 2010; Rodnitzky 1975). The results from studies investigating the effects of long-term OP pesticide exposure on neurobehavioural performance, are therefore inconsistent among adults.

1.2.2. OP pesticides and vibration sensitivity

Vibration sensitivity testing is frequently used in epidemiologic studies to determine the toxic effects of OPs (Holtman 2013; London et al. 1998; Pilkington et al. 2001; Steenland et al. 1994; Stokes et al. 1995). The test is used to assess an individual's peripheral somatosensory function. As with studies that assessed the effect of OP on neurobehavioural performance, inconsistent findings were reported for the studies investigating the association between OP exposure and vibration sensitivity (Holtman 2013; Lee et al. 2003; London et al. 1998).

A study found that farmers exposed to OPs exhibited a non-significant higher mean vibration threshold sensitivity for both the dominant and non-dominant hands compared to the non-exposed individuals (Stokes et al. 1995). Various tests were performed in the study done by Steenland et al. (1994), including neurobehavioural, nerve conducting and vibrotactile sensitivity tests. The results showed that the OP poisoned cases performed significantly worse on vibrotactile sensitivity tests than those not poisoned. Other studies have also shown a decrease in vibration sensitivity among those exposed compared to non-exposed (Cole et al. 1997; Holtman 2013; McConnell et al. 1994).

1.3. The effect of genetic polymorphisms of OP pesticide metabolizing enzymes on neurotoxicity

Evidence has emerged, largely from animal studies, that certain enzymes are capable of detoxifying metabolites of OPs and therefore reducing the risk of harmful outcomes (Costa et al. 1998). Furthermore, genetic polymorphisms or variations of these xenobiotic metabolising enzymes (XMEs) exist in human populations, thereby accounting for variability in metabolising properties among individuals. Paraoxonase (PON1), Glutathione S-transferases (GST) and N-acetyltransferases (NAT) are some of the studied XMEs (Costa et al. 2003a; Costa et al. 2003b; Singh et al. 2011a; Singh et al. 2011b; Singh et al. 2012).

1.3.1. PON1 enzyme

Paraoxonase (PON1) is a calcium-dependent enzyme responsible for hydrolysing the metabolites of several OPs. PON1 is produced in the liver and secreted into the plasma. The enzyme belongs to a family of proteins which includes PON2 and PON3. Unlike PON1, PON2 and PON3 do not have the ability to metabolise OPs (Costa et al. 2003a).

In addition to hydrolysing OPs, PON1 is capable of metabolising a range of lactones and oxidised lipids including both low density and high density lipoproteins (HDL). Because of its multiple biological roles, PON1 plays a role in the pathophysiology of many diseases (Costa et al. 2013).

1.3.1.1. Polymorphisms of the PON1 gene

Genetic studies have shown that PON1 expression varies in the human population (Costa et al. 2005; Eckerson et al. 1983), where PON1 gene activity has been observed to vary between 10-40 fold and PON1 plasma levels may vary up to 13 fold between individuals (Davies et al. 1996; Humbert et al. 1993). Studies have shown that environmental factors, genetic polymorphisms and other factors such as age, sex and nutrition can all affect the levels of PON1 in plasma. However, genetic polymorphisms have been shown to have the biggest effect on variability of PON1 function (Costa et al. 2003b; Ferré et al. 2003; Vincent-Viry et al. 2003).

Two polymorphisms affect the functional activity of PON1, namely, the glutamine (GLN or Q)/arginine (ARG or R) substitution at position 192 (PON1-192Q/R) and the leucine (LEU or L) /methionine (MET or M) substitution at position 55 (PON1-55L/M) (Costa et al. 2003b; Costa et al. 2005). The polymorphism at position 55 is responsible for PON1 levels in plasma and the polymorphism at position 192 determines catalytic efficiency. LEU has been associated with higher PON1 plasma levels when compared to MET at this position. At position 192, the ARG alloform hydrolyses paraoxon more rapidly than GLN. In contrast, GLN hydrolyses OPs; diazinon, sarin and soman at a more rapid rate than ARG does (Costa et al. 2005; Costa et al. 2013).

1.3.1.2. Evidence from animal studies for the effect of PON1 on OP neurotoxicity

Animal studies have supported the hypothesis that PON1 may modify the neurotoxic effects of OP pesticides. Experiments where PON1 serum from rabbits were injected into rats showed that rats who received PON1 and a dose of OPs had significantly less neurotoxic deficits compared to those injected with OPs only (Costa et al. 2003c). Furthermore, a number of studies involving rabbits, rats, mice and birds have shown different degrees of neurotoxic effects in animals with known differences in PON1 levels (Costa et al. 1998).

1.3.1.3. Epidemiological studies investigating the effect of PON1 polymorphisms on OP neurotoxicity amongst workers exposed to pesticides

Four studies that investigated the effect of PON1 on OP toxicity amongst workers exposed to pesticides were identified in the literature (Lee et al. 2003; Mackness et al. 2003; Singh et al. 2011b; Sozmen et al. 2002). These studies, although conducted in different settings, using different study designs and investigating different health outcomes, provide evidence that the susceptibility to the toxic effects of OPs vary among individuals and that the variation is due to the PON1 polymorphism at positions 55 and 192 of the PON1 gene.

A cross-sectional study conducted on farmers in the Western Cape province of South Africa found that farm workers with the GLN/GLN homozygous (Q192Q/Q) or GLN /ARG (Q192R) heterozygote genotypes were almost three times more (OD=2.80; CI: 1.7 – 6.9) likely to report symptoms associated with chronic OP poisoning compared to those with the homozygous ARG/ARG (R192R) genotype at position 192. Furthermore, the prevalence of chronic OP poisoning was the lowest among the non-applicators with the homozygous R192R genotype followed by the non-applicators with the Q192Q or Q192R and the applicators with the R192R genotype. The prevalence of OP poisoning was the highest among the applicators with the Q192R or Q192Q genotype (Lee et al. 2003).

A case-control study by Mackness et al. (2003) in the UK was done to determine the association between PON1 polymorphism and reported chronic ill health, among sheep dipping farmers who were occupationally exposed to OPs particularly, diazoxon. Based on the literature, the Q-contaning alloenzyme hydrolyses diazoxon more rapidly than the R alloenzyme. From the study, more controls had the Q alloenzyme than the cases (60.6% vs 39.7%), showing that individuals with the Q192Q and Q192R amino acid combination were 2.39 (95% CI = 1.46 – 3.98) times more likely to be a 'case' than those with the R192R amino acid combination. Furthermore, individuals with the L55L amino acid combination were 3.16 (95% CI=1.88 – 5.31) times more likely to be a case than those with the L55M and/or M55M amino acid combination.

Sozmen et al. (2002) investigated cases who were poisoned through oral ingestion, injection and with the intention of suicide in Turkey. The study found that PON1 activity was lower in the cases when compared to the controls, (30% lower activity: 114.2 nmol/mL/min vs 152.9 nmol/mL/min) and six months later upon remeasurement, the PON1 levels of the cases increased. Furthermore, cases were more likely to have the Q192 alloenzyme of PON1 polymorphism than controls. Individuals with the Q192 (likelihood ratio = 7.637, P=0.022) and M55 (likelihood ratio=4.721, P=0.094) alloenzymes were more sensitive to OP toxicity than other groups. Lastly, PON1 is an important determinant of OP sensitivity as individuals with mild symptoms of OP poisoning had higher PON1 activity than participants who experienced severe symptoms (Sozmen et al. 2002).

Singh et al. (2011b) conducted a study in India, investigating the effect of PON1 polymorphism on genotoxicity in the form of DNA damage. Exposed participants sprayed OPs for public health programmes and the controls were healthy volunteers. The study found that for both the exposed and control group, the R192R genotype had the higher PON1 activity compared to the Q192Q genotype. Similarly, the L55L genotype showed higher PON1 activity than the M55M genotype. Furthermore, participants with the Q192/Q and M55M genotype had a higher prevalence of DNA damage and were therefore more susceptible to the effects of OPs (Singh et al. 2011b).

1.3.2. Genetic polymorphisms of other xenobiotic metabolizing enzymes

In addition to PON1, Glutathione S-transferases (GST) and N-acetyltransferases (NAT) are XMEs capable of detoxifying OPs (Abhishek et al. 2010; Abel et al. 2004). Four epidemiologic studies have investigated the effect of these XMEs on OP toxicity. Three of the studies were done in India, where DNA damage among OP pesticide workers was investigated (Singh et al. 2011a; Singh et al. 2012). The first study found that DNA damage was significantly higher among workers exposed to OPs (14.37±2.15) compared to controls (6.24± 1.37) and DNA damage was higher among participants with the GSTM1 null genotype compared to those with the GSTM1 positive genotype (15.18 vs. 14.15 tail % DNA, $p = 0.03$). Furthermore, there was no effect of the GSTT1 null genotype on DNA damage (Singh et al. 2011a). The second study found that DNA damage was higher among those with the GSTM1 null genotype and the NAT2 slow acetylators. In addition, mild to severe smokers with the NAT2 slow acetylators were also shown to be more sensitive to the effects of OPs through increased DNA damage (Singh et al. 2012).

A similar study was conducted by Abhishek et al. (2014) in India, investigating the effect of GST (GSTT1 and GSTM1) enzymes on DNA damage among individuals occupationally exposed to pesticides. The study showed that GSTT1 played an important role in OP susceptibility but GSTM1 had no effect on DNA damage. Individuals with the GSTT1 null genotype showed greater levels of DNA damage compared to those with the GSTT1 positive genotype (14.43 vs 9.82, p-value<0.05).

Lastly, the study by Godoy et al. (2014) in Brazil looked at GST polymorphisms (GSTT1 and GSTM1) and pesticide toxicity in individuals who were occupationally exposed to pesticides. Among the participants, 18% had the GSTT1 null genotype, 49% had the GSTM1 null genotype and 10% had both null genotypes. The study found no association between the GST (GSTT1 and GSTM1) polymorphisms and pesticide toxicity. Furthermore, the authors identified personal protection equipment (PPE) as an important determinant in pesticide intoxication.

2. Problem statement

OP pesticide exposure has been associated with acute and chronic neurotoxicity. XMEs such as GST, NAT and PON1 have been shown to detoxify OPs. Laboratory studies have shown that genetic variations exist in these enzymes, causing a variation in their activity in human populations. It is therefore, understandable that some individuals may be more susceptible to the adverse health effects of OPs (Costa et al. 2003a; Costa et al. 2005).

In the literature, no epidemiological studies investigating the effect of XME genetic polymorphisms on long-term OP neurotoxicity could be identified. The few epidemiological studies that have been conducted have focused on outcomes such as DNA damage and acute pesticide intoxication. Furthermore, these studies showed differing results. Singh et al. (2011a) found that the genetic polymorphism of GSTM1 modified OP DNA damage whereas Abhishek et al. (2014) found that GSTT1 played an important role in DNA damage resulting from OP exposure. A few studies have also indicated that PON1 polymorphism modifies OP pesticide toxicity and DNA damage (Lee et al. 2003; Mackness et al. 2003; Singh et al. 2011b; Sozmen et al. 2002). Despite the widespread use of OP pesticides in South African agriculture, only one study was previously conducted in the country (Lee et al. 2003).

The effect of genetic polymorphisms of XMEs on long-term OP neurotoxicity in human population is therefore largely unstudied.

3. Research aim and objectives

3.1. Aim

The aim of this study is to determine whether genetic polymorphisms of XMEs (GST: GSTM1 and GSTT1, NAT2 and PON1: at positions 55 and 192) modify the neurotoxic effects (neurobehavoural performance and vibration sensitivity) of occupational OP pesticide exposure among emerging farmers and farmworkers in the Western Cape of South Africa.

3.1.1. Objectives

- To describe the demographic and socio-economic profile of emerging farmers in the Western Cape
- To determine the long-term exposure to OP pesticides among the emerging farmers and farmworkers
- To measure chronic neurotoxic outcomes among the emerging farmers through the use of neurobehavioural tests and vibration sense testing
- To determine the relationship between long-term exposure to OPs and performance on neurobehavioural tests and vibration sense testing, among emerging farmers and farmworkers
- To determine the prevalence of genetic polymorphisms of XMEs (GST: GSTM1 and GSTT1, NAT2 and PON1: at positions 55 and 192) among emerging farmers and farmworkers
- To determine the effect of XMEs genetic polymorphisms on the relationship between long-term exposure to OPs and performance on neurobehavioural tests and vibration sense testing, controlling for confounders.

3.2. Hypothesis

The hypothesis of this study is that genetic polymorphisms of XMEs modifies the neurotoxicity of OP pesticides among emerging farm workers.

4. Methods

4.1. Study Design, Sampling and Study Population

This cross-sectional study forms part of a larger cohort study investigating the relationship between long-term OP pesticide exposure and neurobehavioural deficits among emerging farmers and farmworkers (Holtman 2013). This study used the baseline data collected by Holtman (2013), to investigate the effect of XMEs genetic polymorphisms on OP neurotoxicity.

The participants were selected from farmer projects registered with the Land Reform Office in South Africa. In 2009 in the Western Cape, 183 emerging farmer projects were registered encompassing a total of 14 624 emergent farmers. Due to ease of accessibility, the study sample was restricted to projects from the following districts: Overberg, Cape Winelands and the West Coast. The farmer projects in these three selected districts were considered to be a representative sample of the projects in the Western Cape (Holtman 2013). Deciduous fruit, vegetables, grapes, cotton, flowers and livestock were produced on the farms.

Of the 63 projects registered in the selected districts, 34 were eligible to participate in the study based on the inclusion/exclusion criteria. Twenty-one farmer projects were approached and agreed to participate in the study. Therefore, 62% of the eligible projects were represented in the study sample.

A total of 326 farmers from the projects who agreed to participate in this study were eligible for inclusion, of whom 316 (97%) agreed to participate. The farmers were interviewed for their demographic information, lifestyle information such as smoking and alcohol consumption, medical history and work information. Blood samples were taken at the beginning of the study to determine genetic variations of the XMEs.

4.2. Inclusion and exclusion criteria

The criteria for inclusion in the study were the following:

- Farms using pesticides
- Farms in one of the three districts (Cape Winelands, Overberg and West Coast)
- Emerging farmers or adult family members
- Males and females 18 years and older

The criteria for exclusion from the study were the following:

- Organic or farms that did not use pesticides or in the process of switching to methods that did not include pesticides
- Hired labourers
- Adults with an abnormality of the lower limbs were excluded from vibration sense testing
- Individuals with a history of the following medical conditions:
    - Encephalitis
    - Tuberculosis Meningitis
    - Stroke, Organic brain syndrome
- Individuals with a history of use of psychotropic medication

4.3. Sample Size Calculation

The sample size calculation was based on the means and variances sourced from studies that assessed neurobehavioural performance of individuals occupationally exposed to OPs (Cole et al. 1997; Kamel et al. 2003; London et al. 1997). Using a significance level of 5% and power of 80%, it was calculated that a sample of 160-350 participants were needed to identify anticipated differences in neurobehavioural tests.

Table 1: Studies assessing neurobehavioural performance of OP occupational exposure

Neurobehavioural outcomes	**Reference study**	**Exposed group mean (SD)**	**Non-exposed group mean (SD)**	**Based on comparing cumulative exposure: 50% prevalence (cumulative exposure cut off at median (1:1)**	**Based on comparing acute episodic exposures over 12 months: 10% incidence (1:9)**
Digit Span	Cole et al. 1997	6.8 (2.0)	7.5 (2.4)	314	75 (n1)
				(157 + 157)	675 (n2)
				Total N = **314**	**750**
	Kamel et al. 2003	4.74 (0.99)	5.63 (1.02)	42	11 (n1)
				(21 + 21)	99 (n2)
				Total N = **42**	**110**
	London et al. 1997	4.8 (1.0)	5.2 (1.0)	198	55 (n1)
				(99 + 99)	495 (n2)
				Total N = **198**	**550**
	Heed pilot 2005	8.7 (3.05)	7.00 (3.07)	244	29 (n1)
				(122 + 122)	261 (n2)
				Total N = **244**	**290**

Neurobehavioural outcomes	Reference study	Exposed group mean (SD)	Non-exposed group mean (SD)	Based on comparing cumulative exposure: 50% prevalence (cumulative exposure cut off at median (1:1)	Based on comparing acute episodic exposures over 12 months: 10% incidence (1:9)
Digit Symbol	Srivasta et al. 2000	51.0 (10.9)	62.4 (13.7)	38	9 (n1)
				(19 + 19)	81(n2)
				Total N = **38**	**90**
	Heed pilot 2005	17.5 (9.1)	13.4 (9.1)	156	43 (n1)
				(78 + 78)	387 (n2)
				Total N = **156**	**430**
	London et al. 1997	25.3 (10.4)	20.9 (9.5)	162	48 (n1)
				(81 +81)	432 (n2)
				Total N = **162**	**480**
	Cole et al. 1997	25.0 (12.4)	22.3 (7.5)	454	196 (n1)
				(227 + 227)	392 (n2)
				Total N = **454**	**588**
Santa Ana	Kamel et al. 2003	18 (3.49)	19.9 (3.04)	94	37 (n1)
				(47 + 47)	74 (n2)
				Total N = **94**	**111**
	London et al. 1997	38.5 (6.9)	35.9 (7.7)	250	90 (n1)
				(125 + 125)	180 (n2)
				Total N = **250**	**270**
	Heed pilot 2005	36 (8.7)	31 (9.3)	102	38 (n1)
				(51 + 51)	76 (n2)
				Total N = **102**	**114**

Neurobehavioural outcomes	Reference study	Exposed group mean (SD)	Non-exposed group mean (SD)	Based on comparing cumulative exposure: 50% prevalence (cumulative exposure cut off at median (1:1)	Based on comparing acute episodic exposures over 12 months: 10% incidence (1:9)
Benton Visual	Cole et al. 1997	8.7 (2.7)	9.8 (2.2)	158	63 (n1)
				(79 +79)	126 (n2)
				Total N = **158**	**189**
	London et al. 1997	6.9 (2.0)	6.1 (2.1)	208	77 (n1)
				(104 +104)	154 (n2)
				Total N = **208**	**231**
	Heed pilot 2005	5.35 (2.58)	4.62 (1.70)	282	120 (n1)
				(141 +141)	240 (n2)
				Total N = **282**	**360**

4.4. Measurement Instruments

4.4.1. Questionnaire

Trained interviewers administered a questionnaire titled the Farmer Questionnaire (see Appendix A) to participants in their spoken language (English and Afrikaans). These questionnaires were back-translated in these languages. The questionnaires included sections on demographic data such as age, sex and education level; information about potential confounders such as alcohol use, smoking, previous head injuries and information regarding current and previous work related exposures to pesticides. Information about previous pesticide poisoning was also collected. The Farmer Questionnaire was successfully piloted in the three districts before the study was conducted.

To determine occupational exposure to pesticides, the details of the number of years and number of days performing applicator (mixing, tractor boom spraying, hand-spraying) and non-applicator activities for the current and 4 previous jobs were obtained from the questionnaires (Appendix A). Details of the pesticides used in these jobs were also obtained to determine if OP pesticides were applied. Additionally, the crops produced on the farms were identified to determine the crop-sector in which a particular job was performed. The total number of days worked in all these jobs was calculated and weighted by job activity using Job-exposure matrices (JEMs). JEMs have been shown to be a repeatable proxy for exposure to pesticides in the Western Cape (London and Myers 1998). The JEM weighted days was further weighted by crop sector to obtain JEM crop weighted days. Exposure intensity was determined by dividing these JEM weighted days and JEM crop weighted days by the number of years worked. These exposure indices were determined for all pesticide exposure as well as OP specific exposures.

4.4.2. Testing for neurotoxicity

In the main study, neurotoxicity was assessed using tests from the WHO Neurobehavioral Core Test Battery (WHO NCTB) and vibration sensitivity testing. The WHO NCTB tests are pen and paper-based tests that are administered orally. These tests are therefore easily administered and have been shown to consistently identify neurotoxicity among individuals who had been occupationally exposed to pesticides. Furthermore, London et al. (1997) have shown that these tests are successfully administered to individuals with low education levels.

Vibration sensitivity threshold test was conducted as studies have shown that OP exposure is associated with impaired vibration sensitivity (Steenland et al. 1994; Stokes et al. 1995). These tests were conducted by trained research assistants in the main study. In this thesis, the results of the Digit Span tests and Vibration tests will be analysed.

4.4.2.1. Digit Span tests

The Digit Span forward and backward WHO NCTB tests assesses the participants' verbal memory and involves an administrator reading out a sequence of numbers to the participant, who then repeats the sequence to the administrator. The aim of the test is to repeat the sequence in the correct order. Initially the administrator reads out a sequence consisting of three numbers and as the test continues, more numbers are added to the sequence, increasing the complexity of the test.

In the Digit Span forward test, the participants are required to repeat the sequence of numbers in the same order as read by the administrator, and evaluates the participant's attention. The Digit Span backward test requires the participant to repeat the sequence of numbers, read by the administrator, in the reverse order. The backward test therefore tests the participant's memory.

For both of the tests, a score of one is given for each sequence correctly repeated. For example, a score of 2 is awarded if the participant repeated both sequences, forward and backward, correctly. In contrast, a score of 0 is awarded when both sequences are incorrectly repeated. Depending on the sequence of numbers, it is possible for an individual to achieve a total score ranging between 0 and 28. Furthermore, the digit span Wechsler Adult Intelligence Scale (WAIS) results will also be included in this study. The digit span WAIS score is the combined score for digit span forward and backward, adjusted for the participant's age and gender.

4.4.2.2. Vibration Sense Threshold

Vibration sensitivity will be measured using a 256-Hz frequency tuning fork on the participant's non-dominant lower limb, while they are seated. After applying the tuning fork, the participant was asked whether he/she felt the vibration sensation and to inform the examiner when the vibration sensation was no longer felt. Three readings will be recorded and the average reading of the last two will be used as the final extinction time for each participant.

4.4.3. Genetic Polymorphism

A qualified nurse drew 10 ml whole blood sample from all participants in the field which was kept at room temperature and then transported to the UCT Human Genetics laboratory on the same day. The genotyping for XMEs; PON1 (at the position 92 and 55), GSTT1, GSTM1 and NAT2 (single nucleotide) was done.

The Puregene® DNA Purification Kit was used to isolate DNA from peripheral blood lymphocytes (Gentra Systems, Minneapolis, USA). The concentration and quality of DNA was assessed by spectrophotometry and agarose gel electrophoresis respectively.

4.5. Statistical analysis

The variables used in the statistical analysis are depicted in Table 2 below.

Table 2: List of variables

	Variable Name	**Type**
DEMOGRAPHIC INFORMATION	Gender	Binary (Male/Female)
	Age	Continuous (Years)
	Language	Categorical (English/Afrikaans/ IsiXhosa/IsiZulu)
	Education	Binary (High:>9 years/Low:≤9 years)
	Type of crop farming	Categorical (Any combination/ Citrus/Deciduous/Grapes/No crops/Other/Vegetables)
	Height	Continuous (Metres)
	Weight	Continuous (Kilograms)
	BMI	Continuous
	Alcohol Consumption	Binary (Yes/No)
	Smoking status	Binary (Yes/No)
	Head Injury	Binary (Yes/No)
	Psychiatric Illness	Binary (Yes/No)
PESTICIDE EXPOSURE	Pesticide Applicators (all pesticides and OP pesticide)	Binary (Yes/No)

	Variable Name	Type
	Days worked unweighted by activity or crop sector (all pesticides and OP pesticide)	Continuous (Days)
	Days worked weighted by JEM (JEM days) for all pesticides and OP specific pesticides	Continuous (Days)
	Days worked weighted by JEM and crop sector (JEM crop days) both for all pesticides and OP specific pesticides	Continuous (Days)
	Intensity of exposure: JEM days and JEM crop days divided by number of years worked	Continuous (Days)
	JEM days (weighted by JEM) for all pesticides and OP specific pesticides	Categorical (non-exposed/≤25th percentile/≤50th percentile/≤75th percentile/≥75th percentile)
	Diagnoses of past pesticide poisoning	Binary ((Yes/No)
XENOBIOTIC METABOLISING ENZYMES	GSTT1	Binary (Null/Yes)
	GSTM1	Binary (Null/Yes)
	NAT2	Categorical (GG/GA/AA)
	PON1_55	Categorical (TT/TA/AA)
	PON1_192	Categorical (GG/GA/AA)
NEUROBEHAVIOURAL OUTCOMES	Digit Span Forward	Continuous (Test score)
	Digit Span WAIS	Continuous (Test score)
	Vibration Sensitivity	Continuous (Test score)
	Digit Span Forward	Categorical (High:≥median/Low: ≤median)
	Digit Span WAIS	Categorical (High:≥median/Low: ≤median)
	Vibration Sensitivity	Categorical (High:≥median/Low: ≤median)

From the above, there are eleven exposure variables, three outcome variables and five effect modifiers, the XMEs (GSTT1, GSTM1, NAT2, PON1_55 and PON1_192)

4.5.1. Exploratory data analysis

To describe the sample population exploratory data analysis, univariate and multivariate (mostly bivariate) analysis will be done. For the univariate analysis, histograms to determine the distribution and summary statistics will be calculated for the continuous variables. The categorical variables will be explored using frequency tables. Furthermore, box-plots will be created to identify any outliers in the data. Associations between two continuous variables will be explored through scatter plots and box plots will be created to explore associations between continuous and categorical variables. Contingency tables will be used to identify associations between two categorical variables.

Either the two-tailed t-test for normally distributed continuous variables or the Wilcoxon rank sum test for continuous non-normal variables will be used to compare the means of continuous variables. The Fisher exact test or the chi-squared test will be used to determine the bivariate associations between categorical variables.

4.5.2. Multivariate Analysis

All statistical analysis will be performed using the statistical software package STATA 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). Linear and logistic multiple regression analysis will be used to determine the relationship between pesticide exposure and neurobehavioural outcomes. Two sets of models will be created for each outcome, exposure and genetic variable combination. The first set of models will show the relationship between OP exposure and neurobehavioural outcomes adjusted for confounders and the second set of models will include the gene-exposure interaction term. Confounders will be identified from literature and variables found to have significant ($p \leq 0.05$) association with neurobehavioural outcomes in the bivariate analysis. Models showing significant interactions will be stratified by polymorphism categories.

5. Ethics

This cross-sectional study is a sub-study of the study titled *Neurobehavioural effects of pesticide exposure among emerging farmers in the Western Cape* by Holtman (2013), which has received ethical approval by the Research Ethics Committee, Health Sciences Faculty of the University of Cape Town (REC REF: 477/2007). The purpose of the study was clearly explained to potential participants, verbally and in their predominant language. A study information sheet (see Appendix B) was used and this was done before consent to participate was obtained. Written informed consent (see Appendix C) was obtained in accordance with the requirements of the Helsinki Declaration and the Medical Research Council of South Africa guidelines (World Medical Association (WMA) 2000; South African Medical Research Council (MRC) 1993). Consent forms were provided in English and Afrikaans.

Furthermore, safety training with materials developed by the Centre for Environmental and Occupational Health Research Unit (CEOHR), UCT were provided to participants as a form of compensation. Any participants who were identified with neuropsychological disorders or any untreated injuries were referred to local health care providers.

Anonymity was achieved through the use of study numbers rather than participants' names. Furthermore, confidentiality was maintained as the research team only had access to the data and group results were reported.

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PART B: Structured Literature Review

1. Introduction

1.1. Background and Objectives of literature review

Worldwide organophosphate (OP) pesticides, first synthesised in the 1940's, are the most widely used chemical class of pesticides (Costa et al. 2005a; Mackness et al. 1997; Singh et al. 2011a). The OP pesticides are used for pest control in various sectors such as agriculture, horticulture and in domestic and commercial settings. South Africa is the largest consumer of pesticides in sub-Saharan Africa and in the Western Cape Province the production of fruit and wine are important for export and therefore, for economic growth (Naidoo and Buckley 2003).

OPs have been associated with a wide range of negative health effects including neurodegenerative diseases such as Parkinson's and Alzheimer's disease (Baldi et al. 2003; Le Couteur et al. 1999; Freire and Koifman 2012), cancers such as Non-Hodgkin's lymphoma, leukemia and multiple myeloma (Mills and Zahm 2001), respiratory problems and asthma (Faria et al. 2005; Hoppin et al. 2008; Hoppin et al. 2009) and depression and anxiety (Mackenzie Ross et al. 2010).

There is evidence that xenobiotic metabolising enzymes (XMEs) such as Glutathione S-transferases (GST), N-acetyltransferases (NAT2) and Paraoxonase (PON1) are capable of detoxifying metabolites of OPs (Costa et al. 2003a; Singh et al. 2011a; Singh et al. 2012). Furthermore, the evidence indicates that among humans, the activity of these enzymes are determined by genetic polymorphisms. Therefore, susceptibility to the harmful effects of OPs may vary in human populations (Costa et al. 1999; Costa et al. 2003a; Costa et al. 2005a; Singh et al. 2011b; Singh et al. 2012; Lee et al. 2003; Mackenzie Ross et al. 2010).

The objectives of this literature review were to: i) provide an overview of the neurotoxic effects of OP pesticides, ii) review studies investigating the neurotoxic effects associated with long-term OP pesticide exposure in humans, iii) identify genetic polymorphisms of XMEs that modify OP neurotoxicity in human populations and iv) review epidemiological studies investigating the effect of XMEs genetic polymorphisms on OP neurotoxicity.

1.2. Search strategy

For the first two objectives, the most recent reviews and textbooks were consulted.

For objectives 3 and 4, the following search strategy was used: literature published between 1975 and September 2015 were searched for using the electronic search tools: PubMED (http://www.ncbi.nlm.nih.gov/pubmed/), MEDLINE (https://www.nlm.nih.gov/bsd/pmresources.html) and Google Scholar (https://www.google.co.za/webhp?sourceid=chrominstant&ion=1&espv=2&ie=UTF-8#q=google%20scholar). References of the identified articles were checked for any unidentified literature and relevant articles suggested by these search engines were also considered. Furthermore, only laboratory and epidemiological studies investigating genetic polymorphisms of the following XMEs were included in the review: glutathione S-transferases (GSTT1 and GSTM1), N-acetyltransferases (NAT2) and Paraoxonase (PON1).

For the epidemiological studies, studies on all populations exposed to OP pesticides including: men, women or children and farm and non-farm populations were included in the review. Studies from all developing and developed countries that were available, and all study designs were included in the review.

1.3. Search terms

Search terms for objective 1 and 2 included:

Pesticide, organophosphate, organophosphorous, OP, neurotoxic, neurobehavioural, neurobehavioral and health effects of pesticides

Search terms for objective 3 and 4 included:

Genetic polymorphism, OP pesticides, Paraoxonase, PON, PON1, Glutathione S-transferases, GST, N-acetyltransferases, NAT, xenobiotic metabolising enzymes and genetic susceptibility.

2. Neurotoxicity of organophosphate pesticides

OP pesticides are neurotoxic, capable of affecting the central and peripheral nervous system (Costa et al. 2005). Depending on the level of exposure, OPs can result in four distinct neurological syndromes: (1) acute cholinergic effects, (2) Intermediate Syndrome (IMS), (3) Organophoshorous-induced delayed neurotoxicity (OPIDN) and lastly, (4) organophosphorus ester-induced chronic neurotoxicity (OPICN).

2.1. Acute cholinergic effects

Acute cholinergic effects including salivation, sweating, muscle twitching, reduced consciousness, bronchial secretion and constriction of the bronchi, arise after an acute single dose of exposure to OPs. In extreme instances acute cholinergic effects can manifest as seizures and respiratory failure due to paralysis of the diaphragm. These symptoms may arise as soon as a few hours, or as late as five days after exposure, and it is possible to recover from these adverse cholinergic effects (Costa et al. 2003c; Major 2010).

The symptoms of this syndrome arise due to the accumulation of the neurontransmitter, acetylcholine at the nerve endings. Exposure to OPs inhibit acetylcholinesterase (AChE), an enzyme that inactivates acetylcholine. The inhibition of AChE causes an accumulation of acetylcholine which results in the over stimulation of postsynaptic cholinergic receptors and the disruption of the transmission of neuron impulses, thus leading to the common cholinergic effects (Alavanja et al. 2004; Costa et al. 2005a).

2.2. Intermediate syndrome (IMS)

IMS is characterised by weakness of the neck and eye muscles, proximal skeletal and respiratory muscles which may last for as long as 6 weeks. The syndrome occurs 1-4 days following acute OP intoxication and after the acute cholinergic effects. Between 20%-50% of poisoned cases result in IMS. The mechanism that causes IMS is not yet well known, but individuals with this type of neurotoxicity can recover from the symptoms within 3 weeks (Balali-Mood and Saber 2012; Major 2010).

2.3. Organophoshorous induced delayed neurotoxicity (OPIDN)

The OPIDN toxicity occurs after IMS, 4 weeks after either a large single dose of OPs or repeated exposures to OPs. The syndrome is characterised by general muscle weakness and pain including initial paraesthesia and calf pain, weakness in the distal leg muscles resulting in foot drop, and claw hand caused by weakening of the muscles in the hands (Costa et al. 2005a; Major 2010).

Recovery from OPIDN can take as long as 12 months and may not be complete. Although the mechanism of this syndrome is unclear it is thought that it occurs due to the phosphorylation of neuropathy target esterase (NTE) (Costa et al. 2003a; Lotti and Moretto 1999).

2.4. Organophosphorus ester-induced chronic neurotoxicity (OPICN)

OPICN is characterised by neurobehavioural, neurological and neuropsychological symptoms caused by exposure to either a large single dose of OP or long-term repeated low OP exposure. Symptoms therefore include cognitive dysfunction, difficulty concentrating, tremors, lack of motor control, generalized weakness, impairment of visual memory, a decrease in verbal attention, anxiety and depression. Unlike the other syndromes, the effects of OPICN can continue years after the OP exposure (Stallones and Beseler 2002; Savage et al. 1988).

Neurotoxicity due to long-term OP pesticide exposure includes effects on neurobehavioral performance and effects on vibration sense (Holtman 2013).

2.5. Neurobehavioural performance and long-term exposure to OP pesticides

A recent review included several studies that investigated the association between long-term OP pesticide occupational exposure and neurobehavioural performance, and found that the findings of these have not been consistent (Ismail et al. 2012). For instance, a cross-sectional study in south-eastern Spain showed that exposure to OPs leads to lower neuropsychological performance among pesticide applicators.

The applicators performed worse on the Benton Visual Form Recognition Tests (OR=6.93, 95% CI: 1.52– 31.51), when compared to the reference group. This test assesses visual perception and memory (Roldán-Tapia et al. 2005). Another cross-sectional study done on adolescents occupationally exposed to OPs found that the applicators performed significantly worse on neurobehavioural tests of memory and attention span compared to the non-applicators (Rohlman et al. 2014). However, a cohort study done found that occupational exposure to the OP pesticide, chlorpyrifos, had no effect on peripheral neuropathy and no association with nerve conduction study (NCS) (Albers et al. 2004).

The lack of accurate exposure measurement, small sample sizes, differences in neurobehavioural measurement and study designs have been described as the causes for the inconsistent results produced (Ismail et al. 2012).

Three meta-analysis were conducted to determine the effects of long-term OP exposure. The first was done by Ismail et al. (2012) on 17 studies and the second done by Ross et al. (2013) on 14 studies between the years 1960 and 2012. The more recent study was done by Meyer-Baron et al. (2015) on 22 eligible studies, between 1965 and 2010. Individuals were generally exposed through OP pesticide application and manufacturing. These meta-analyses showed that OP exposed individuals performed worse on attention and memory tests compared to the non-exposed group.

Furthermore, reviews by Colosio et al. (2009) and Rohlman et al. (2011) also found that the majority of the studies showed an association between OP exposure and neurobehavioural deficit among individuals occupationally exposed.

These reviews also showed that studies that focused on the neurobehavioural effects associated with OP poisoning have produced consistent results (Colosio et al. 2009). For instance, a cross sectional study on farmers in China who had been poisoned in the last 12 months, showed that participants performed significantly worse on WHONCTB (digit span forward and backward, digit symbol, Benton visual retention, correct pursuit aiming and error pursuit aiming scores) compared to non-poisoned individuals (Zhang et al. 2016). A study conducted in Florida, on individuals occupationally exposed to OPs for at least 1 month, showed that OPs negatively impacted performance on digit span (OR = 1.90; CI 1.02-3.53), tapping (coefficient = 4.13; 95% CI, 0.00-8.27), Santa Ana test (coefficient = 1.34; 95% CI, 0.29-2.39) and postural sway (coefficient = 4.74; 95% CI, -2.20 to 11.7) tests (Kamel et al. 2003).

Three studies investigating the effects of cumulative exposure to OP pesticides and neurobehavioural performance have been conducted in South Africa, all of which were in the Western Cape Province (Holtman 2013; London et al. 1997; Major 2010). All the studies found no association between OP exposure and neurobehavioural performance. The comparison group in these studies were farm workers who may have been exposed to OPs. London et al. (1997) focused on deciduous fruit farm workers, Major (2010) on grape farmers and Holtman (2013) on emerging farmers in the Western Cape Province. Two of the three studies were cross sectional studies. Major (2010) used the general health questionnaire (GHQ) and the Brief Symptom Inventory (BSI) to determine neurotoxic outcomes associated with OP exposure, but found no association among the 817 South African fruit farmers. London et al. (1997) found a very small association between Pursuit-Aiming and Santa Ana tests, but no association with the 5 other tests from the WHO Neurobehavioral Core Test Battery (WHO NCTB).

The only cohort study in SA was conducted by Holtman (2013) on 319 emerging farmers. Neurobehavioural performance was assessed using a combination of tests from the WHO NCTB, Brief symptom inventory (BSI) and the Swedish Q16. The study found no significant association between long-term OP exposure and neurobehavioural performance, but found that individuals that experienced pesticide poisoning in the past performed worse on the digit span forward test, which tests memory and attention (OR 2.67; 95% CI 1.05 - 6.80).

2.5.1. OP pesticides and vibration sensitivity

Few studies have investigated the effect of OP exposure on peripheral somatosensory function, decreased vibration sensitivity. A review of studies investigating the association between OP exposed workers and vibration sensitivity have shown inconsistent results (Holtman 2013). OP applicators in the Agricultural Health Study (AHS) showed decreased toe vibration sensitivity compared to non-applicators (Starks et al. 2012). A study done by Steenland et al. (1994) also found associations between OP exposure and decreased vibration sense. A cross-sectional study in Eucador found that applicators had a lower toe vibration threshold compared to the control group (Cole et al. 1997). However, Stokes et al. (1995) found a non-significant higher mean vibration threshold sensitivity for both the dominant and non-dominant hands compared to the general population.

Three studies have been conducted in South Africa. Two have found significant impairment in vibration sensitivity among the exposed group (Holtman 2013; London et al. 1998). Manjra et al. (ND) found a decrease in vibration sense among the OP applicators and Holtman (2013) found impaired vibration sense among emerging farmers. However, London et al. (1998) found no association between OP exposure and impaired vibration sensitivity among fruit farmers in the Western Cape Province.

3. Genetic polymorphism of XMEs

3.1. Glutathione S-transferases (GST)

GST, which are also present in plants and bacteria, have been shown to play an important role in insect resistance to insecticides including OPs (Clark 1989; Fournier et al. 1992; Reidy et al. 1990; Wei et al. 2001). In humans, there are four distinct classes of GST including, alpha (A), mu (M), pi (P) and theta (T) and these are distributed to different tissues. In human populations, the polymorphism of the Mu (GSTM1) and Theta (GSTT1) classes are well documented. The polymorphisms occur due to gene deletions resulting in null genotypes. The Individuals with the null genotypes are suspected to be more sensitive to effects of OPs due to its reduced metabolising properties (Abel et al. 2004).

3.2. N-acetyltransferases (NAT2)

The two N-acetyltransferases that are polymorphic in human populations are called NAT1 and NAT2. These enzymes are involved in the detoxification of a variety of aromatic amine and hydrazine drugs. The enzyme NAT2 was discovered before NAT1, and the NAT2 polymorphism was identified when the differences in isoniazid toxicity among TB patients was noted. In addition to detoxifying various drugs, N-acetyltransferases are carcinogen and OP metabolising enzymes, and due to their polymorphic distribution in humans, it is postulated that some individuals may be more susceptible to the harmful effects of these exposures than others. Several epidemiologic studies have linked variants in NAT2 to urinary, bladder, colorectal, breast and lung cancer (Grant et al. 1997; Hein et al. 2000; Hein 2002).

3.3. Paraoxonase (PON1)

Paraoxonase (PON1) is a calcium-dependent enzyme responsible for hydrolysing the metabolites of several OPs and a range of lactones and oxidised lipids (HDL and LDL) (Costa et al. 2013). The enzyme is named 'paraoxonase' because it hydrolyses paraoxon which is the active metabolite of parathion, one of the most studied OPs (Costa et al. 2013). In humans, PON1 is produced in the liver and secreted into the plasma. The enzyme belongs to a family of proteins which also includes PON2 and PON3. Unlike PON1, PON2 and PON3 do not metabolise OPs (Costa et al. 2005a).

Laboratory studies have shown that PON1 activity in the human population (Costa et al. 2005a; Eckerson et al. 1983) vary between 10-40 fold (Humbert et al. 1993) and PON1 plasma levels may vary up to 13 fold between individuals (Davies et al. 1996). The variability occurs due to environmental factors such as age, sex and nutrition, and due to polymorphisms on the PON1 gene. Genetic polymorphisms have been found to have the biggest effect on variability of enzyme levels between individuals (Ferré et al. 2003; Vincent-Viry et al. 2003).

Animal studies have shown that PON1 may modify the neurotoxic effects of OP pesticides (Costa et al. 1999; Costa et al. 2003b). A number of studies involving rabbits, rats, mice and birds have shown different degrees of neurotoxic effects in animals with known differences in PON1 levels (Costa et al. 1999). Further evidence from an experiment where PON1 serum from rabbits were injected into rats, showed that rats who received PON1 and a dose of OPs had significantly less neurotoxic deficits compared to those injected with OPs only (Costa et al. 2003a).

Two polymorphisms affect the activity of PON1, the glutamine (GLU)/arginine (ARG) substitution at position 192 (PON1-192Q/R) and the leucine (LEU)/methionine (MET) substitution at position 55 (PON1-55L/M). The LEU/MET ploymorphism is responsible for PON1 levels in plasma and the GLU/ARG polymorphism determines catalytic efficiency. Leucine has been associated with higher PON1 plasma levels when compared to methionine, but the efficiency of the GLU/ARG polymorphism is substrate specific. Arginine has been shown to hydrolyse paraoxon more rapidly, while glutamine metabolises diazinon, sarin and soman at a more rapid rate (Costa et al. 2005a; Furlong et al. 2005).

3.4. Effect of XME polymorphisms on OP toxicity

Table 1 summarises epidemiological studies that have investigated the effect of XMEs (GST: GSTT1 and GSTT1, NAT2 and PON1: PON1-55 and PON1-192) polymorphisms on OP pesticide neurotoxicity and DNA damage. There were four studies investigating PON1 polymorphism and four investigating the GST and NAT2 polymorphisms.

3.5. Epidemiological studies investigating the effect of PON1 polymorphisms on OP neurotoxicity

A cross-sectional study conducted on farmers in the Western Cape Province of South Africa found that subjects with the PON1-192Q/Q or PON1-192Q/R genotypes were almost three times more likely to report symptoms that were associated with chronic OP poisoning compared to those with the PON1-192R/R genotype (CI: 1.7 – 6.9). Furthermore, the prevalence of chronic OP poisoning appeared to be the lowest among the non-applicators with the PON1-192R/R genotype, followed by the non-applicators with the PON1-192Q/Q and the PON1-192Q/R genotype. The prevalence of OP poisoning was the highest among the applicators with the PON1-192Q/Q or the PON1-192Q/R genotypes (Lee et al. 2003).

A case-control study by Mackness et al. (2003) was conducted to determine the association between PON1 polymorphisms and reported chronic ill health among sheep dipping farmers. These farmers were from the United Kingdom and occupationally exposed to OPs, particularly diazoxon. From the literature the 192Q alloenzyme hydrolyses diazoxon more rapidly than the R alloenzyme and in this study, more controls had the Q alloenzyme than the cases (60.6% vs 39.7%). The results showed that individuals with the PON1-192 QQ and the PON1-192 QR genotype were 2.39 (95% CI = 1.46 – 3.98) times more likely to be a case than those with the PON1-192 RR genotype. Furthermore, individuals with the PON1-192 LL genotype were 3.16 (95% CI=1.88 – 5.31) times more likely to be a case than those with the PON1-192 MM polymorphism.

A study done in Turkey by Sozmen et al. (2002) investigated the effect of OP poisoning on PON1. Cases were poisoned through oral ingestion, injection and with the intention of suicide. The study found that PON1 activity was lower in the cases when compared to the controls, (30% lower activity: 114.2 nmol/mL/min vs 152.9 nmol/mL/min). Six months later, when measured again, the PON1 levels of the cases increased. Furthermore, cases were more likely to have the Q alloenzyme for PON1 polymorphism at position 192 than controls. Individuals with the Q (likelihood ratio = 7.637, P=0.022) and M (likelihood ratio=4.721, P=0.094) alloenzymes for polymorphisms at position 192 and 55 respectively, were more sensitive to OP intoxication than other groups. PON1 is an important determinant of OP sensitivity as individuals with mild symptoms of OP poisoning had higher PON1 activity than participants who experienced severe symptoms.

Lastly, a cross sectional study conducted in New Delhi, India by Singh et al. (2011b) investigated the effect of PON1 polymorphism on genotoxicity in the form of DNA damage. Exposed participants sprayed OPs for public health programmes and the controls were healthy volunteers. The study found that for both the exposed and control group, the individuals with the PON1-192 RR genotype had the higher PON1 activity compared to those with the PON1-192 QQ polymorphism. Similarly, the participants with the PON1-55 polymorphism showed higher PON1 activity than those with the PON1-55 MM genotype. Furthermore, participants with the PON1-192 QQ and PON1-55 MM genotypes had a higher prevalence of DNA damage and were therefore more susceptible to the effects of OPs.

Although all studies provided evidence that PON1 polymorphisms modify OP toxicity, the study by Mackness et al. (2003) found the PON1-192 RR and the PON1-55 LL genetic variants to be the 'slow' metabolisers, which is in contrast to the other studies. The three earlier studies (Lee et al. 2003; Mackness et al. 2003; Sozmen et al. 2002) investigated poisoning as a health outcome and not chronic neurotoxicity; and Singh et al. (2011b) focused on DNA damage. There is, therefore, no data from epidemiological studies on the effect of PON1 polymorphism on OP chronic neurotoxicity. Furthermore, no cohort studies were conducted and the exposure variables were categorical (applicator status). Cohort studies using more sensitive outcome of chronic neurotoxicity and exposure measurements are therefore required.

3.6. Epidemiological studies investigating the effect of GST and NAT polymorphisms on OP neurotoxicity

In addition to PON1, GST (GSTM1 and GSTT1) and NAT2 are XMEs capable of detoxifying OPs (Abhishek et al. 2010; Godoy et al. 2014; Singh et al. 2012). Four epidemiologic studies have investigated the effect of these XMEs on OP toxicity. Two of the studies were done by Singh et al., who investigated DNA damage among OP pesticide workers (Singh et al. 2011a; Singh et al. 2012). The first study found that DNA damage was significantly higher among workers exposed to OPs (14.37±2.15) compared to controls (6.24± 1.37) and DNA damage was higher among participants with the GSTM1 null genotype compared to those with the GSTM1 positive genotype (15.18 vs. 14.15 tail % DNA, p = 0.03). Furthermore, there was no effect of the GSTT1 null genotype on DNA damage and OP exposure (Singh et al. 2011a). The second study found that DNA damage was higher among those with the GSTM1 null genotype and in NAT2 slow acetylators. Furthermore, mild to severe smokers who were NAT2 slow acetylator, were also shown to be more sensitive to the effects of OPs, through increased DNA damage (Singh et al. 2012).

A similar study was conducted by Abhishek et al. (2014) in India, investigating the effect of GST (GSTT1 and GSTM1) enzymes on DNA damage among individuals occupationally exposed to pesticides. The study showed that GSTT1 played an important role in OP susceptibility, but that GSTM1 had no effect on DNA damage. The individuals with the GSTT1 null genotype showed higher DNA damage compared to those with the GSTT1 positive genotype (14.43 vs 9.82, p-value<0.05).

Lastly, the study by Godoy et al. (2014) in Brazil looked at pesticide intoxication and GST polymorphisms (GSTT1 and GSTM1). Participants were occupationally exposed to pesticides. Among the participants, 18% had the GSTT1 null genotype, 49% the GSTM1 null genotype and 10% had both null genotypes. The study found no association between the GSTT1 and GSTM1 polymorphisms and pesticide intoxication. Instead personal protection equipment (PPE) was reported to be an important determinant in pesticide intoxication (Godoy et al. 2014).

Only one of the studies investigated the effect of GST polymorphisms on OP toxicity, but found no evidence of effect modification (Godoy et al. 2014). The remaining studies showed that GST played a significant role in OP toxicity modification, but DNA damage was investigated as a health outcome and not chronic neurotoxicity. There is therefore little data from epidemiological studies on the effect of GST and NAT2 polymorphisms on OP chronic neurotoxicity.

In addition to few studies been conducted, the findings of the existing studies are inconsistent. This could be due to chance, differences in study designs or due to different measurement of the exposure. Furthermore, the studies were conducted in different settings, the differences in genetic background could therefore have also contributed to the difference in findings.

Table 3: Summary of epidemiological studies investigating the effect of XMEs genetic polymorphisms on OP neurotoxicity AND genotoxicity

Author, year	Population (n), Study design and Setting	OP exposure assessment	Outcome variable	Genotype assessment	Findings
Studies investigating PON1 polymorphism					
Sozmen et al. 2002	Case control study of 94 subjects (28 OP poisoned patients and 66 healthy volunteers) Izmir Turkey.	Patients admitted to emergency service at Ataturk Research and Educational Hospital in October 1999 – July 2000. Route of OP poisoning included oral (n=26), injection (n=1) and inhalation (n=1).	Acute OP intoxication	PON1 at position 192	The study found that PON1 activity was lower in the cases when compared to the controls, (30% lower activity: 114.2 nmol/mL/min vs 152.9 nmol/mL/min) and six months later, the PON1 levels of the cases increased. Furthermore, cases were more likely to have the Q alloenzyme for PON1 polymorphism at position 192 than controls. Individuals with the Q (likelihood ratio = 7.637, P=0.022) and M (likelihood ratio=4.721, P=0.094) alloenzymes for polymorphisms at position 192 and 55 respectively, were more sensitive to OP intoxication than other groups.
Lee et al. 2003	A cross sectional study of 100 farm workers from Western Cape Province, South Africa.	Applicator status.(applicator vs non-applicator) Subjects were matched by characteristics such as age.	OP chronic toxicity (subjects with two or more symptoms associated with OP chronic toxicity, self-reported)	PON1 at position 192	Having one of the either Gln/Gln or Gln/Arg genotypes independently predicted an increased risk of neurotoxic symptoms (OR 2.9, 95% CI 1.7-6.9). Furthermore, the prevalence of chronic toxicity increased in a stepwise fashion from 15.0% among pesticide non applicators with a "fast metabolism" (Arg/Arg) genotype, to 42.9% among pesticide non applicators with "slow metabolism" (Gln/Gln or Gln/Arg) genotypes, to 58.8% among pesticide applicators with "fast metabolism" genotype, and 75.0% among pesticide applicators with "slow metabolism" genotypes (P=0.001).
Mackness et al. 2003	A case control study of 396 sheep dippers in the UK.	OP exposure while sheep dipping	Self-reporting of chronic ill health.	PON1 at positions 192 and 55.	Cases were found to be more likely to have the R192 allele and the L55 allele when compared to the controls. A combination of the R (position 192) and the L (position 55) genotypes was associated with lower PON1 activity towards diazoxon in both cases and controls.
Singh et al., 2011b	This cross sectional study consisted of 230 participants. The study took place in New Delhi, India.	The number of hours applying OP pesticides in all jobs was determined for all participants. Those with more than 2400 h were classified as exposed.	DNA damage (Tail moment) as a proxy for genotoxicity. DNA damage was evaluated using the alkaline comet assay.	The PON1 genotypes at position 192 were determined by PCR amplification using previously described primers.	The results revealed that PON1 activity toward paraoxon was significantly lower in workers than in control subjects (179.19±39.36 vs. 241.52±42.32 nmol/min/ml). The DNA damage was observed to be significantly higher in workers than in control subjects and the individuals with PON1 Q/Q and M/M genotypes (slow genes) showed significantly higher DNA damage compared to other isoforms (pb0.05).

Author, year	Population (n), Study design and Setting	OP exposure assessment	Outcome variable	Genotype assessment	Findings
Studies investigating GST and NAT2 polymorphisms					
Singh et al. 2011a	A cross-sectional study with 230 participants conducted in New Delhi, India.	An index was calculated for each interviewed subject (based on their hours/day×days/year×years). Subjects with an exposure index of more than 2400 h were classified as exposed. The non-exposed group had less than 2400 h OP exposure.	DNA damage (Tail moment) as a proxy for genotoxicity. DNA damage was evaluated using the alkaline comet assay.	An individual multiplex PCR (polymerase chain reaction) was carried out to determine GST genotyping. Globin gene was used as an internal control	The first study found that DNA damage was significantly higher among workers exposed to OPs (14.37±2.15) compared to controls (6.24± 1.37) and DNA damage was higher among participants with the GSTM1 null genotype compared to those with the GSTM1 positive genotype (15.18 vs. 14.15 tail % DNA, p = 0.03). There was no effect of the GSTT null genotype on DNA damage
Singh et al. 2012	A cross-sectional study of 230 participants conducted in New Delhi, India.	An index was calculated for each interviewed subject (based on their hours/day×days/year×years). Subjects with an exposure index of more than 2400 h were classified as exposed.	DNA damage (Tail moment) as a proxy for genotoxicity. DNA damage was evaluated using the alkaline comet assay.	An individual multiplex PCR (polymerase chain reaction) was carried out to determine GST genotyping. Globin gene was used as an internal control	The second study found that DNA damage was higher among those with the GSTM null genotype and the NAT slow acetylators. Furthermore, mild to severe smokers with the NAT2 slow acetylators were also shown to be more sensitive to the effects of OPs, through the increased DNA damage (Singh et al, 2012).
Abhishek et al. 2014	A cross-sectional study of 67 participants conducted in Punjab, India	The exposed group consisted of 40 workers exposed to various pesticides and a group of 27 unexposed control agricultural workers with an average age of 36.1 and 38.4 years, respectively.	Damage Index (DI) and Damage Frequency (DF), were used to measure DNA damage. They were determined by summing up the visual score of 100 cells of each individual. Furthermore, % DNA in tail was also used to measured DNA damage. % DNA in tail was measured using the computerized image analysis software (TriTek CometScore).	Genotyping of the GST enzymes was done using multiplex PCR described by Arand et al. 1996	DNA damage was significantly more prevalent among the exposed group compared to the unexposed. Furthermore, Individuals with the GSTT1 null genotype showed greater levels of DNA damage compared to those with the GSTT1 positive genotype (% DNA tail 14.43 vs 9.82, p-value<0.05).
Godoy et al. 2014	A cross-sectional study of 235 farm workers, from the Goias municipalities Brazil.	Applicator status (Applied/ not applied pesticides)	Number of events of OP intoxication	GSTT (The quantitative polymerase chain reaction (qPCR) method was used)	Found no association between GSTT1 and GSTM1 null polymorphisms and intoxicated events. Found an association between PPE and events of intoxication and therefore presents the importance of PPE use.

4. Conclusion

There is consistent evidence in international literature that a history of OP pesticide poisoning causes neurotoxicity among farm workers but inconsistent evidence that long-term pesticide exposure, controlling for pesticide poisoning, causes neurotoxicity (Holtman 2013). Only three studies have been conducted in South Africa, one of them a cohort study with one year of follow-up (Holtman 2013; London et al. 1997; Major 2010). Furthermore, none of these studies found an association between long-term OP exposure and neurobehavioural performance.

Few cross-sectional and case control have investigated the effect XMEs genetic polymorphisms on health outcomes associated with long-term OP pesticide exposure (Lee et al. 2003; Sozmen et al. 2002; Singh et al. 2011a; Singh et al. 2011b; Singh et al. 2012). The studies that have been conducted provide some evidence that the genetic polymorphisms of XMEs may modify OP toxicity. However, the types of effect in these studies are inconsistent. Exposure indices in these studies were not sensitive and none investigated neurotoxic outcomes apart from poisoning and DNA damage.

Future research requires more cohort studies investigating the effect of XMEs polymorphisms on long-term OP pesticide exposure neurotoxicity. Furthermore, these future studies should use more sensitive OP exposure measurements and outcome instruments in different settings, including South Africa.

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PART C: Journal Ready Manuscript

This article has been prepared for the purposes of submission to the Journal, *Environment Health Persepctive*. The *Instructions to Authors* document has been attached (Appendix E). The author adhered to all the instructions set out by the Journal, however, for the purpose of this thesis, some tables are included in the text.

Title: Genetic Polymorphisms and Organophosphate Neurotoxicity among Emerging farmers in the Western Cape

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Thesis abstract

Thesis abstract

BACKGROUND: Long-term exposure to organophosphates (OPs) can cause chronic neurotoxic effects which may be modulated by genetic polymorphisms of xenobiotic metabolising enzymes (XMEs). No previous study investigated XME modulation of neurotoxicity outcomes.

OBJECTIVES: To investigate whether XMEs polymorphisms modulate OP neurotoxicity among emerging farmers.

METHODS: A cross-sectional study of 301 emerging farmers was conducted in the rural Western Cape of South Africa. Neurotoxicity testing included the World Health Organisation Core Test Battery (digit span forward and backward) and vibration sensitivity testing. Questionnaire items included demographic data, potential confounders and work history of pesticide exposures. Blood samples were analysed for genetic polymorphisms of the following XMEs; glutathione S-transferases (GST), N-acetyltransferases (NAT) and Paraoxonase (PON1).

RESULTS: Median age was 39 (30-48) and most had 9 years of education or less (65.5%). 54% of the participants were OP pesticide applicators. There was a low prevalence of the GST null genotype (GSTT-1% and GSTM-16%) and the GA and GG genotype for NAT (10%). Modulation of OP exposure and neurotoxic outcome relationships by NAT, PON1 at position 192 and GST was indicated in multivariate analysis. The strongest evidence of modification was by NAT on the relationship between pesticide poisoning and impaired vibration sense. Poisoned individuals with the GG genotype were more likely to suffer from impaired vibration sense compared to GA and AA genotypes.

CONCLUSION: Genetic polymorphisms of NAT, PON1 (at position 192) and GSTM may modify the relationship between OP exposure and neurotoxicity. This however requires further exploration in larger longitudinal studies and preventive strategiesto reduce pesticide exposure amongst vulnerable groups should be implemented.

1. Introduction

Worldwide, organophosphates (OPs) are the most widely used class of pesticide (Costa et al. 2003; Mackness et al. 1997; Singh et al. 2011a). Exposure to OP pesticides have been associated with a wide range of neurotoxic outcomes such as neurodegenerative diseases (Parkinson's disease and Alzheimer's disease) (Baldi et al. 2003; Freire and Koifman 2012; Le Couteur et al. 1999), impairment in memory and concentration (Abdel Rasoul et al. 2008; Bouchard et al. 2011), depression and anxiety (Mackenzie Ross et al. 2010) and negative effects on the peripheral somatosensory function (Stephens et al. 1995; Stokes et al. 1995). Results of studies investigating long-term OP neurotoxicity have however been inconsistent, with some studies showing no association between outcomes and exposure and others showing an association (Godoy et al. 2014; Holtman 2013).

Evidence from animal studies suggest that susceptibility to the toxicity of OPs may be influenced by xenobiotic metabolising enzymes (XMEs) (Costa et al. 1990; Gan et al. 1991; Wei et al. 2001). XMEs are enzymes capable of detoxifying metabolites of several OPs. These XMEs include glutathione S-transferases (GST), N-acetyltransferases (NAT2) and Paraoxonase (PON1) (Costa et al. 2003; Singh et al. 2011a; Singh et al. 2011b; Singh et al. 2012). Furthermore, the catalytic efficiency of these enzymes have been shown to be determined by their genetic polymorphisms or variations in human populations (Abdel Rasoul et al. 2008; Humbert et al. 1993).

Two polymorphisms affect the activity of PON1, the glutamine (GLU)/Arginine (ARG) substitution at position 192 (PON1-192Q/R) and the leucine (LEU)/methionine (MET) substitution at position 55 (PON1-55L/M) (Costa et al. 2005). The LEU/MET polymorphism is responsible for PON1 levels in plasma and the GLU/ARG polymorphism determines catalytic efficiency. Leucine has been associated with higher PON1 plasma levels when compared to methionine, but the efficiency of the GLU/ARG polymorphism is substrate specific. Arginine has been shown to hydrolyse the organophosphate paraoxon more rapidly, while glutamine metabolises diazinon, sarin and soman at a more rapid rate (Costa et al.1999; Costa et al. 2003).

Although very few epidemiological studies have been conducted to determine the effects of the polymorphism on OP toxicity, all the studies reported the importance of PON1 and its polymorphism on OP susceptibility (Lee et al. 2003; Leng and Lewalter 1999; Mackness et al. 2003; Singh et al. 2011b; Sozmen et al. 2002).

Furthermore, these studies were conducted in different settings, used different study designs and investigated different health outcomes. The studies were done on individuals exposed to OPs through sheep dipping in UK (Mackness et al. 2003), poisoning through suicide attempts (either orally or through an injection) in Turkey (Sozmen et al. 2002), OP sprayers for public health programmes in India (Singh et al. 2011b) and South African farm workers (Lee et al. 2003). However, none of these studies investigated neurobehavioural performance as an outcome of interest.

In addition to PON1, GST and NAT2 are XMEs capable of detoxifying OPs (Abel et al. 2004; Abhishek et al. 2010; Singh et al. 2012). In humans, there are four distinct classes of GST: alpha (A), mu (M), pi (P) and theta (T). Genetic polymorphisms of the mu (GSTM1) and theta (GSTT1) group exist and are well documented in literature (Abhishek et al. 2010). The variation in these enzymes are due to gene deletions resulting in null genotypes. Individuals with the null genotypes are suspected to be more susceptible to effects of OPs due to its reduced metabolising properties (Abel et al. 2004; Singh et al. 2011a).

Four epidemiological studies were conducted to determine whether GST (GSTM1 and GSTT1) and NAT2 modifies OP neurotoxicity and the results differ between these studies. In India, Abhishek et al. (2014) found that GSTT1 played an important role in OP susceptibility but GSTM1 had no effect on DNA damage. The OP exposed individuals, with the GSTT1 null genotype showed higher DNA damage compared to those with the GSTT1 positive genotype (% DNA in tail 14.43 vs 9.82, p-value<0.05). In contrast, Godoy et al. (2014) found no association between the GST (GSTT1 and GSTM1) polymorphisms and pesticide intoxication among individuals occupationally exposed to OPs in Brazil.

The remaining two studies investigated DNA damage among OP pesticide applicators (Singh et al. 2011a; Singh et al. 2012) and found that GSTM1 played a significant role in OP susceptibility, as DNA damage was higher among individuals with the GSTM1 null genotype. GSTT1 and NAT2 had no effect on DNA damage caused by OP exposure. However, OP exposed individuals with the concomitant GSTM1 and GSTT1 null genotypes experienced higher levels of DNA damage than those with the positive genotypes. Similarly, DNA damage was higher among those with the GSTM1 null genotype and the NAT2 slow acetylators (Singh et al. 2011a; Singh et al. 2012).

Even though South Africa is the largest consumer of pesticides in sub-Saharan Africa, only one study has investigated the effect of xenobiotic enzymes, PON1, on OP neurotoxicity in the country (Lee et al. 2003; Naidoo and Buckley 2003). Furthermore, no previous epidemiological studies have investigated the effect of XMEs genetic polymorphisms on performance on neurobehavioural tests and vibration sensitivity. The aim of the present study was to determine whether the genetic variations of the xenobiotic enzymes (GST: GSTM1 and GSTT1, NAT2 and PON1: PON1-55 and PON1-192), modulate the relationship between long-term OP exposure and neurobehavioural outcomes and vibration sensitivity, amongst emerging farmers in the rural Western Cape of South Africa.

2. Materials and methods

2.1. Study design, population and sampling

This cross-sectional study formed part of a larger cohort study investigating the neurobehavioural effects of occupational OP pesticide exposure on emerging farmers in the Western Cape, South Africa (Holtman 2013). This sub-study used data collected at baseline in the cohort study to investigate the effect of genetic polymorphisms of XMEs on neurotoxicity resulting from long-term exposure to OPs. This study received ethical approval by the Research Ethics Committee, Health Sciences Faculty of the University of Cape Town (HREC REF 386/2015).

In 2009, we recruited participants from three (Overberg, Cape Winelands and the West Coast) of the six districts in the Western Cape. This was done by contacting farmer projects registered with the Land Reform Office in South Africa. The crops produced on the farms were representative of that produced in the Western Cape (Holtman 2013). Furthermore, the farmer projects in these districts had not switched to organic methods of crop production. Lastly, the three selected districts were located close to Cape Town which facilitated data collection.

Of the 34 eligible registered farmer projects in the selected districts, 21 agreed to participate in the study. The 21 farmer projects housed 326 farmers and farm workers, all of whom agreed to participate. Written informed consent was obtained in English or Afrikaans from each individual before the study commenced.

2.2. Demographic and confounder information

All participants were asked to complete a questionnaire in their preferred language, administered by a trained interviewer. The questionnaire (Appendix A) consisted of standard demographic information (gender, age, language and education level), illnesses (any previous head injuries and psychiatric illnesses), lifestyle factors (alcohol consumption, smoking and other drugs) and socioeconomic information (possession of the following household appliances: television, electricity, computer and a telephone landline).

2.3. Exposure information

The work history section of the questionnaire comprised of questions on the current and four previous occupations (occupation type and number of years worked). If the work was performed on a farm, there were further questions on: type of crop produced on the farms, job title, tasks or activities performed, the number of days doing general farm work or applying specific pesticides (mixing, spraying) and the use of personal protective equipment (PPE). The cumulative number of days worked was determined retrospectively. The participants were questioned on how many days per week, weeks per month and months per year worked. Furthermore, the number of years worked were also recorded and the numbers of days worked (8 hour days), and therefore days exposed to pesticides, calculated. The exposure days were then weighted using a Job-exposure-matrix (JEM), which was adapted from the matrix previously developed for a study conducted on farm workers in the rural Western Cape. The JEM measurement has been shown to be repeatable in settings like the rural Western Cape (London and Myers 1998).

The exposure indices generated using the JEM included: the accumulated number of working days weighted by job activity (JEM days), JEM days weighted by crop usage in 2009 (JEM crop days) and JEM and crop days per annum of work life. The exposure measurement per annum was used as an index of the intensity of occupational exposure to OP pesticides. The pesticide exposure measurements were weighted for the following tasks: mixing pesticides indoors or outside, applying

pesticides with a backpack, spraying pesticides with a tractor using hand-directed or a quad bike (Mixing indoors = 1.00, Mixing outdoors = 0.80, Tractor spraying with mist blower or boom sprayer = 0.70, Backpack spraying = 0.70 and Quadbike spraying = 0.70). All other tasks were given a weighting of zero.

2.4. Neurobehavioural assessment

Previous studies have shown that cumulative exposure to OPs can cause neurobehavioural deficits such as impairment of one's attention, memory and concentration (Bouchard et al. 2011; Abdel Rasoul et al. 2008). Therefore, neurobehavioural performance was assessed using two tests drawn from the World Health Organisation Neurobehavioral Core Test Battery (WHO NCTB), digit span forward and digit span backward. The two-digit span tests assess both attention and memory; and are pen and paper-based tests that are conducted orally. They are therefore easily used in developing countries and have been shown to consistently identify neurotoxicity among exposed individuals (London et al. 1997). Furthermore, the totals of the two tests were combined and standardised (for the participants' age and gender) to form the digit span Wechsler Ault Intelligence Scale (WAIS) score. From Holtman (2013), 43% of the studies that used the digit span forward test found a positive association between the test and OP exposure. In addition, digit span WAIS was also positively associated with exposure measurements. These two tests, digit span forward and digit span WAIS, were therefore used to assess neurotoxicity in this study.

OP exposure can also lead to decreased peripheral somatosensory function. Previous studies have confirmed this and shown an association between OP exposure and reduced vibration sense (Steenland et al. 1994; Stokes et al. 1995). We therefore conducted vibration sense testing in addition to the neurobehavioural tests, in this study. We used a 256-Hz frequency tuning fork to measure vibration sensitivity; by applying it to the participant's non-dominant lower limb.

2.5. Polymorphisms in XME genes

DNA was isolated from peripheral blood lymphocytes of individuals with the Puregene® DNA Purification Kit (Gentra Systems, Minneapolis, USA) according to the manufacturer's specifications. Standard DNA quality control measures included spectrophotometry for quantification and agarose gel electrophoresis for integrity determination. DNA stock samples were stored at -80°C for the long-

term and retrieved from storage for genotyping assays. For the amplification of the PON1rs854560 (p.Leu55Met) and PON1*rs662 (p.Gln192Arg)*, 100ng of template DNA was included in a final polymerase chain reaction (PCR) volume of 25µl containing; 1x GoTaq buffer (Promega®), 200µM of each deoxynucleotide triphosphate (dNTPs), 0.5 units of GoTaq DNA polymerase and 0.4µM of each oligonucleotide. The DNA template was denatured at 95°C for 5 min and amplified for 30 cycles consisting of 94°C for 30sec, Ta for 30sec and 72°C for 40sec.

A final step at 72°C for 7min was included to complete the extension of all DNA fragments. The PCR for all polymorphisms were completed on the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, Foster City, CA, USA). The PCR fragment containing the PON1 polymorphism rs854560 (p.Leu55Met) was digested with NlaIII (New England Bio Labs, Cambridge, UK). Similarly, PON1 polymorphism rs662 (p.Gln192Arg) were genotyped by the digestion of PCR fragments with AlwI (New England Bio Labs). All the resulting products were separated on an 3% (w/v) SeaKem® LE Agarose (Lonza, Basel, Switzerland) gel and visualized with the nucleic acid stain, ethidium bromide (0.5µg/ml; Sigma-Aldridge, St. Louis, MO, USA).

The null alleles for GSTT1 and GSTM1 were assayed by co-amplification of the β-Globin gene [3]. In this assay 100ng of template DNA is amplified in a final reaction volume of 25µl containing 0.4µM of each primer for β-Globin and GSTT1/ GSTM1. The amplified products for the GSTT1 assay were separated on a 1% (w/v) SeaKem® LE Agarose (Lonza) gel, whilst GSTM1 was assayed on a 2% agarose (Lonza) gel and visualized with the nucleic acid stain, ethidium bromide (0.5µg/ml; Sigma-Aldridge).

The NAT2 polymorphism, rs1799931 p. Gly286Glu, was genotyped by digestion of the PCR fragment with BamHI (New England Bio Labs) and subsequent visualization of the products on a 2% (w/v) agarose (Lonza) gel. PCR fragments containing rs1799931 were amplified from 100ng template DNA in a final reaction volume of 25µl containing 0.4µM of each primer.

3. Statistical Analysis Section

Univariate (histograms and contingency tables) and bivariate (box plots, scatter plots and two tailed t-test) exploratory analysis was performed. All outcome variables (vibration sensitivity, digit span forward and WAIS) were continuous and transformed to binary variables using the median value as the cut-off value (≤ median). Therefore, both linear and logistic regression was used to determine the effect of genetic polymorphisms on OP neurotoxicity. Exposure variables included pesticide and OP pesticide applicator status; and continuous pesticide and OP exposure variables (JEM days, JEM crop days and JEM crop days per annum). The continuous OP exposure variables were transformed to a five level categorical variable consisting of a non OPapplicator category and four categories based on the quartiles of the OP JEM days of the applicators.

Due to the small number of participants with the AA genotype for both NAT and PON1-192, the participants with the AA and GA genotypes were grouped together and compared to those with the GG genotype. Similarly, MM and LM genotypes for the PON1-55 were grouped together. These groupings may differ to that in previous studies. This is largely due to the different study settings, which results in different dominant and non-dominant alleles. If too few participants have a particular allele, the two non-dominant groups are then grouped and compared to the dominant allele.
All analyses were conducted using the statistical software package, STATA 13 (StataCorp. 2013. Stata Statistical Software: Release 13. College Station, TX: StataCorp LP). The confounders were identified in two ways; based on *a priori* and variables with a significant ($p \leq 0.05$) association with the outcome and exposure variables. The confounders identified from the literature were: gender, age, education, language, smoking, psychiatric illness, current alcohol consumption, the CAGE score, previous head injury and low socioeconomic status (London et al. 1998; Steinweg and Worth 1993).

To test for XMEs effect modification, exposure-gene interaction terms were created by multiplying the genetic variables with the exposure variables; OP applicator and the five level categorical OP JEM day variables. These interaction variables along with the genetic variables, were included in the model. Furthermore, the models were stratified by the different categories of the five XMEs (GSTM1, GSTT1, NAT2, PON1-55 and PON1-192) to identify any vulnerable genetic groups.

Diagnostics tests were used to assess model fit and sensitivity analysis. The tests showed that the results do not change when classifying the participants who could not identify the pesticides they were exposed to, as either exposed, non-exposed or missing.

4. Results

4.1. Participation

Most of the participants were from the Cape Winelands district (n=148, 49.2%) followed by the West Coast district (n=84, 27.9%) (Table 1). Of the 326 participants that agreed to participate, 7 did not complete the questionnaire and outcome assessment at study commencement. Furthermore, 11 participants did not provide blood samples and an additional 7 participants were excluded from the analysis due to poor DNA amplification or because the DNA could not be located. Therefore, 301 (92.3%) of the participants eligible to participate in the study was retained.

4.2. Demographic and socioeconomic characteristics

Most of the participants were males (n=204, 67.8%), Afrikaans speaking (n=262, 87.0%) and the median age was 39 (30-48) years (table 1). Approximately two thirds of the participants smoked and/or consumed alcohol. Less than 12.0% of the participants reported previous head injuries and 1.7% reported having a psychiatric illness. About 13.0% owned 3 or less items (television, landline telephone, cellphone, fridge or electricity) and were classified as having the lowest socioeconomic status of the participants.

4.3. Neurobehavioural outcomes and Genetic polymorphisms

The distribution of neurobehavioural performance scores and XMEs genetic polymorphisms are shown in Table 2. The scores for digit span forward and digit span WAIS varied over a narrow range (5-7 and 6-8, respectively) while vibration sensitivity was more variable (9.5-16.5).

Less than 20.0% of the participants had the GST null genotypes; GSTT1 (1.3%) and GSTM1 (16.0%). Few participants had the MM genotype for PON1-55 (<3.0%) and AA genotype for NAT2 (<1.0%) and PON1-192 (11.0%). The GG genotype was predominant for both NAT2 (90.4%) and the PON1-192

polymorphism (45.9%). About 70.0% of the participants had the LL genotype for PON1 polymorphism at position 55.

Table 4: Demographic, Lifestyle and Socioeconomic factors

Variable			N	%
Demographic Variables	Gender	Male	204	67.8
		Female	97	32.2
	Age (years)		39 *	30.0-48.0 **
	Language	English	0	0
		Afrikaans	262	87.0
		Xhosa	31	10.3
		Zulu	0	0
		Sotho	8	2.7
	Education	≤9 years education	197	65.5
		>9 years education	104	34.6
	District	Overberg	69	22.9
		Cape Winelands	148	49.2
		West Coast	84	27.9
Lifestyle Factors	Alcohol Consumption	Current Alcohol consumption	188	62.5
	Smoking Habits	Current Smoker	201	66.8
Injuries and Illnesses	Previous Head Injuries	Reported Head Injury	35	11.6
	Psychiatric Illness	Reported Illness	5	1.7
Socioeconomic Status	Owns ≤3 items		37	12.3

Items for the socioeconomic performance: television, landline telephone, cell phone, fridge or electricity

* median in years

** Interquartile range in years

Table 5: Descriptive information of neurobehavioural outcomes and genetic polymorphisms

Neurobehavioural Outcomes		**Median (IQR)**
Digit Span Forward score		6.0 (5.0-7.0)
WAIS Digit Span score		6.0 (6.0-8.0)
Vibration Sensitivity score		13.0 (9.5-16.5)
Gene variables	**Polymorphism**	**N (%)**
GSTT1	NULL	4 (1.3)
	YES	297 (98.7)
GSTM1	NULL	48 (16.0)
	YES	253 (84.1)
NAT2	GG	272 (90.4)
	GA	27 (9.0)
	AA	2 (0.7)
PON1-55	LL	209 (69.4)
	ML	84 (27.9)
	MM	8 (2.7)
PON1-192	GG	138 (45.9)
	GA/AG	130 (43.2)
	AA	33 (11.0)

4.4. Occupational and pesticide exposure information

Of the 301 participants, approximately 54.0% of the participants were pesticide applicators in their previous or current employment and 22.3% were current OP applicators. About 94.0% (n=283) of the participants worked on a farm and of these workers, almost half were pesticides applicators (53.2%). The rest were farm workers who worked in the field as non-applicators (38.9%) or as general workers (2.0%). Of the participants that did not work on a farm (n=18), less than 1.0% worked in the industry, 3.0% had other forms of employment, 2.0% were pensioners and less than 1.0% were unemployed.

Almost 12.0% reported having been diagnosed with pesticide poisoning in the past and more than 50% of the pesticide applicators worked 543 days, while the OP applicators worked a total of 308 days.

Furthermore, of the past and current pesticide applicators (n=141), less than 1.0% had the GSTT1 null genotype and 14.0% had the GSTM1 null genotype. Among the current OP applicators (n=67), 1.5% had the GSTT1 null genotype and 16.4% had the GSTM1 null genotype. All the participants that were diagnosed with previous pesticide poisoning (n=22) did not have the GSTT1 null genotype, but 9.1% had the GSTM1 genotype.

4.5. Multivariate relationship between OP pesticides exposure and performance on neurobehavioural tests

The multivariate results for the association between pesticide exposure and neurotoxic outcomes, adjusted for confounders and effect modification by polymorphisms in XME genes, using continuous and categorical outcomes and exposure variables, were essentially the same. Therefore, the results of the logistic regression analysis are presented in the paper, as these associations were the strongest.

The logistic regression analysis results for the association between neurotoxic outcomes and OP pesticide exposure are presented in Table 3. Table 3 shows that the only significant association between OP pesticide exposure and neurotoxic outcome were: a) between vibration sense testing and past pesticide poisoning and b) between vibration sense and OP applicators with JEM exposure days between the 50^{th}-75^{th} percentile. The individuals previously poisoned were 3.2 fold more likely to have a low score for vibration sense testing compared to those not poisoned. Furthermore, the performance on the vibration sense test worsens with increased exposure to OPs in a dose dependent manner (OP JEM days percentiles: $<25^{th}$: OR=1.1; CI=0.4-3.2, 25^{th}-50^{th}: OR=2.3; CI=0.7-7.1, 50^{th}-75^{th}: OR=2.8; CI=0.9-8.2), except for those with OP JEM days greater than the 75^{th} percentile (OR=0.6; CI=0.2-1.8). The association between vibration sensitivity and OP JEM days between the 50^{th} and 75^{th} percentile was near significance.

4.6. Multivariate relationship between OP pesticides exposure and performance on neurobehavioural tests modified by XMEs

A summary of the logistic regression analysis results for the association between neurotoxic outcomes and OP pesticide exposure, adjusted for confounders, modified by XMEs polymorphisms is presented in Table 4. The models were selected based on the following criteria: a) a change in the odds ratio for the exposure variable in the model containing XME interaction terms compared to the model that does not include the XME interaction terms and b) significant interaction terms.

Table 4 shows that the PON1 polymorphism at position 192 may modify the relationship between OP applicator and performance on digit span forward. The odds ratio changed from a negative association, 0.7 (95% CI: 0.4 - 1.2) to 1.3 (95% CI: 0.6 – 3.0) after including the interaction term for PON1-192. Similarly, NAT2 may modify the relationship between past pesticide poisoning and vibration sensitivity as the odds ratio changed from 3.2 (95% CI: 1.1 - 9.1) to a statistically significant 4.7 (95% CI: 1.3 – 16.9), after adjusting for the genetic polymorphism. However, none of the XMEs modified the relationship between the exposure variables and the outcome digit span WAIS. Table 4 also shows that GSTM1 may modify the relationship between OP JEM days and vibration sensitivity testing. The odds ratio for the participants between the 50^{th} and 75^{th} percentile was 2.8 (95% CI: 0.9 - 8.2) but changed to a statistically significant 3.1 (95% CI: 1.0 – 9.6), when including the GTSM1 genetic polymorphism.

Table 5 shows the relationship between OP pesticide exposure and neurotoxicity stratified by XME genetic polymorphisms, for the models in Table 4. The stratified results show that OP applicators with the GG genotype for the PON1-192 polymorphism performed worse on the digit span forward than the non OPapplicators with the same genotype (OR=1.1, 95% CI: 0.4-2.8). Although the associations were not significant, the OP applicators with the GA or AA genotype performed better on the digit span forward test compared to the nonOP applicators (OR=0.4, 95% CI:0.2-0.8). Furthermore, previously poisoned participants with the GG NAT2 genotype were 5.7 (95% CI: 1.4 – 22.7) times more likely to have impaired vibration sense compared those not poisoned, whereas previously poisoned participants with the GA or the AA NAT2 genotype were less likely to have impaired vibration sense compared to those not previously poisoned (OR=1.5, 95% CI: 0.1 – 43.2).

There were too few participants that had the null GSTM1 genotype, but for those that did not have the null genotype there were negative associations between vibration sense and OP JEM days quartile exposure groups compared to the base group. For the latter, the exposure response relationship increased in a dose dependent manner until the 75th quartile.

Table 6: Multiple Logistic Regression Analysis for the association between neurobehavioural outcomes and OP JEM days adjusted for confounders (n =301)

		Adjusted associations			Significant covariates in the model	
Dichotomised outcomes:	**Exposure variable:**	**Odds ratio**	**95% CI**	**p-value**	**Covariate**	**OR (95% CI)**
Digit Span Forward	OP applicator	0.7	0.37 - 1.21	0.18	None	
Digit Span WAIS	OP applicator	0.5	0.23 - 1.08	0.08	Education	2.42 (1.20 - 4.89)
Vibration Sense Test	OP applicator	1.4	0.78 - 2.51	0.26	Head Injury	0.36 (0.16 - 0.82)
Digit Span Forward	Past pesticide poisoning	2.1	0.83 - 5.22	0.12	None	
Digit Span WAIS	Past pesticide poisoning	1.5	0.53 - 4.26	0.44	None	
Vibration Sense Test	Past pesticide poisoning	3.2	1.10 - 9.07	**0.03**	Head Injury	0.08 (0.02 - 0.41)
Digit Span Forward	< 25thpercentile	0.8	0.29 - 2.32	0.70	None	
	Between 25th-50th percentile	0.9	0.30 - 2.61	0.82		
	Between 50th-75th percentile	0.2	0.07 - 0.84	**0.03**		
	> 75th percentile	1.0	0.36 - 2.93	0.95		
Digit Span WAIS	< 25thpercentile	0.5	0.11 - 1.98	0.30	None	
	Between 25th-50th percentile	0.8	0.19 - 2.93	0.68		
	Between 50th-75th percentile	1.0	Omitted, predicts failure perfectly			
	> 75th percentile	1.3	0.41 - 4.05	0.67		
Vibration Sense Test	< 25thpercentile	1.1	0.40 - 3.15	0.83	Head Injury	0.41 (0.18 - 0.94)
	Between 25th-50th percentile	2.3	0.71 - 7.13	0.17		
	Between 50th-75th percentile	2.8	0.94 - 8.23	**0.06**		
	> 75th percentile	0.6	0.18 - 1.75	0.32		

		Adjusted associations			Significant covariates in the model	
Dichotomised outcomes:	**Exposure variable:**	**Odds ratio**	**95% CI**	**p-value**	**Covariate**	**OR (95% CI)**
Base exposure group for OP JEM days: 0 days (non-applicators), OP applicator: 1 = applicator, 0 = non applicator; past pesticide poisoning: yes = 1, no =0.						

Table 7: Summary of Multiple Logistic Regression Analysis models for the association between neurobehavioural outcomes and OP JEM days adjusted for confounders, XME polymorphism and XME effect modification with the strongest associations (n =301)

			Adjusted associations			Significant covariates in the model	
Gene	**Dichotomised outcomes:**	**Exposure variable:**	**Odds ratio**	**95% CI**	**p-value**	**Covariate**	**OR (95% CI)**
PON1_192	**Digit Span Forward**	OP applicator	1.3	0.56 - 2.98	0.55	None	
		GA/AA	1.6	0.96 - 2.82	0.07		
		Exposure-gene interaction	0.3	0.09 - 0.92	**0.04**		
NAT	**Vibration sense**	Past pesticide poisoning	4.7	1.28 - 16.94	**0.02**	Head Injury	0.1 (0.015 - 0.38)
		GA/AA	0.6	0.19 - 1.83	0.36		
		Exposure-gene interaction	0.5	0.07 - 2.95	0.41		
GSTM	**Vibration Sense Test**	< 25thpercentile	1.2	0.42 - 3.34	0.75	Head Injury	0.4 (0.18 - 0.94)
		Between 25th-50th percentile	2.5	0.77 - 7.92	0.13		
		Between 50th-75th percentile	3.1	1.02 - 9.60	**0.05**		
		> 75th percentile	0.7	0.21 - 2.27	0.54		
		Null	2.5	0.82 - 7.70	0.11		
		Exposure-gene Interaction	0.7	0.41 - 1.33	0.31		
Base exposure group for OP JEM days: 0 days (non-exposed participants); for OP applicator: non applicator = 0, applicator = 1 Base XME_genetic groups: GSTM: Yes, NAT and PON1_192: GG and PON1 at position 55: LL							

Table 8: Summary the relationship between OP pesticide exposure and neurotoxic outcomes stratified for XME polymorphism for the models listed in Table 4 (n =301)

			Adjusted associations		
Gene	**Neurotoxic Outcomes:**	**Exposure variable:**	**Odds ratio**	**95% CI**	**p-value**
PON1_192 = GG	**Digit Span Forward**	OP applicator	1.3	0.55 – 3.08	0.55
PON1_192 = GA/ AA	**Digit Span Forward**	OP applicator	0.4	0.15 – 0.82	**0.02**
NAT = GG	**Vibration Sense Test**	Past pesticide poisoning	5.7	1.41 – 22.70	**0.02**
NAT = GA/AA	**Vibration Sense Test**	Past pesticide poisoning	1.5	0.05 – 43.18	0.83
GSTM = Yes	**Vibration Sense Test**	< 25thpercentile	1	0.32 - 3.13	0.99
		Between 25th-50th percentile	1.8	0.54 - 6.09	0.34
		Between 50th-75th percentile	3.3	0.95 - 11.44	0.06
		> 75th percentile	0.8	0.23 - 2.59	0.68
GSTM = Null	**Vibration Sense Test**	< 25thpercentile	0.9	0.03 - 27.56	0.98
		Between 25th-50th percentile	1	Omitted	
		Between 50th-75th percentile	2.7	0.15 - 49.74	0.5
		> 75th percentile	1	Omitted	
Base exposure group for OP JEM days: 0 days (non-exposed participants); for OP applicator: non applicator = 0, applicator = 1					

5. Discussion

The results of the study indicate effect modification by PON1-192, NAT2 and GSTM1. There was a strengthened association between OP pesticide exposure and the neurotoxic outcomes, digit span forward and vibration testing (Table 5), after accounting for the genetic polymorphisms of these enzymes.

Firstly, low digit span forward scores were more prevalent among applicators compared to non-applicators among individuals with the GG genotype for the PON1 polymorphism at positon 192. This indicates that neurotoxicity increases with OP exposure for this group of workers. This relationship was not seen among the individuals with the AA and GA genotypes. Therefore, the individuals with the GG genotype at position 192 on the PON1 gene, may be more sensitive to OP toxicity. This result is consistent with the laboratory studies that have shown that the AA genotype hydrolyses paraoxon more rapidly than the GG genotype (Costa et al. 2003; Costa et al. 2005).

The finding is also consistent with three of the previous observational studies conducted, one of them conducted in the Western Cape, of South Africa (Lee et al. 2003; Mackness et al. 2003). The cross-sectional study done in South Africa showed that the participants with the homozygous PON1-192 GG or heterozygous PON1-192 GA genotypes were almost three times more likely to report symptoms that were associated with chronic OP poisoning compared to those with the homozygous PON1-192 AA genotype (CI: 1.7 – 6.9). The case-control study by Mackness et al. (2003) found that individuals with the PON1-192 GG and PON1-192 GA amino acid combination were 2.39 (95% CI = 1.46 – 3.98) times more likely to reporting chronic ill health than those with the PON1-192 AA combination among sheep dipping farmers in the United Kingdom occupationally exposed to OPs. Furthermore, a cross sectional study conducted in New Delhi, India by Singh et al. (2011b) among OP sprayers for public health programmes and controls found participants with the 192GG genotype had a higher prevalence of DNA damage (Singh et al. 2011b).

The second set of evidence for XMEs effect modification is the strengthened relationship between pesticide poisoning and impaired vibration sensitivity when adjusting for NAT2 polymorphism. Furthermore, stratifying by the genetic polymorphism of NAT2 showed a stronger positive relationship between OP poisoning and impaired vibration sensitivity for those with the GG genotype compared to those with the GA and AA genotype. This finding is consistent with that of Singh et al. (2012) who investigated DNA damage among OP pesticide and found that DNA damage was higher among those with the NAT GG genotype. However, it should be noted that few farmers had the AA and the GA genotype in our study (less than 10%).

The third finding in our study indicating effect modification by XMEs, was the reduced vibration sensitivity, due to increased OP exposure (as measured by JEM days), among those with the GSTM1 positive genotype compared to those with the null genotype. This is in contrast to the finding from three of the four studies that investigated the effect of GST on OP neurotoxicity. The studies found that the GSTM1 null genotype was associated with increased DNA damage (Abhishek et al. 2014; Godoy et al. 2014; Singh et al. 2011b; Singh et al. 2012). The low prevalence of the GTSM1 (16%) null genotype found in our study could have produced spurious findings.

This study did not find any evidence of effect modification by the genetic polymorphisms of GSTT1 and PON1-55. Previous studies investigating the role of the GST enzymes have produced inconsistent results. Abshishek et al. (2010) found that GSTT1 modified OP exposure and DNA damage and Singh et al. (2011a; 2012) found no association with GSTT1. Again, the prevalence of GSTT1 (<2%) in this population was low. Previous epidemiological studies have however found that the PON1 polymorphism at position 55, played a role in the modification of OP neurotoxicity. In Turkey, participants with the PON1-M55 genotype were more likely to suffer from OP intoxication (likelihood ratio=4.721, P=0.094) (Sozmen et al. 2002) and in India the OP sprayers with the PON1-L55 genotype showed higher PON1 activity and therefore lower prevalence of DNA damaged (Singh et al. 2011a). However, OP exposed sheep dippers in the UK with the PON1-L55 genotype were approximately 3 (95% CI=1.88 – 5.31) times more likely to be a case, of reported chronic ill health, compared to those with the PON1-L/M55 and the PON1-M55 genotypes (Mackness et al. 2003).

Also, for most of the neurobehavioural outcomes, this study did not find evidence of effect modification by PON1-192, NAT2 and GSTM1. The low prevalence of some of the OP genotypes in the population of emerging farm workers in our study might have affected the findings on effect modification. Less than 2% of the participants had the GSTT1 null genotype and there was therefore not enough variability in the sample to determine the effects of its genetic polymorphism on OP neurotoxicity. Furthermore, with NAT2 and PON1 (at positions 55 and 192), too few participants had the AA genotype (<1%, <3% and 11% respectively). We were therefore unable to compare the performance of these participants to the other genetic groups. In the analysis, individuals with the AA genotype for NAT2 and PON1 (192) were grouped together with the GA participants and then compared to the participants with the GG polymorphism.

Similarly, with the PON1-55 polymorphism, participants with MM and LM genotypes were grouped together and their results compared to those with the LL genotype. Therefore, in this study, it was not possible to determine the individual effects of each genotype.

To our knowledge, this is the first study to look at the effects of genetic polymorphisms on the relationship between long-term OP pesticide exposure and neurobehavioural performance. Previous studies have investigated the effect of XMEs genetic polymorphisms on the relationship between OP pesticide poisoning and outcomes such as such as DNA damage (Lee et al. 2003; Singh et al. 2011a).

The only significant association between long-term pesticides exposure and a neurotoxic outcome in our study, was an association between past pesticide poisoning and performance on the vibration threshold test for those with the GG genotype for NAT. The poisoned individuals showed a significant decrease in vibration sensitivity. Previous studies have consistently shown negative neurobehavioural effects associated with OP poisoning (Colosio et al. 2009) but not consistently with an OP exposure index that does not specifically focus on poisoning. In our study, there was a borderline significant negative association ($p = 0.06$) between vibration sense and OP JEM days in the 3rd quartile and dose response relationship below the 4th quartile for those with the positive GSTM genotype.

In addition to the low prevalence of some XMEs genotypes, the most important limitations of the study were the OP exposure characterisation, reliance on self-reported information and the study design. Long-term pesticide exposure information is particularly difficult to measure as the measurement is reliant on participant's recollection of past pesticide use which may be subject to recall bias. Including pesticide bio-monitoring in the study may enhance exposure estimation.

Information on confounders such as alcohol consumption and smoking may be subject to a desirability bias, where individuals report to not have smoked or consumed alcohol, when they have. The measurement of these confounders are particularly important as alcohol consumption can influence performance on the neurobahvioural tests, and smoking may be linked to neuropathy (National Institute of Neurological Disorders and Stroke 2016), therefore influencing vibration sensitivity. However, the desirability bias is unlikely associated with OP exposure.

Lastly, the study design was a cross-sectional one. We could therefore only measure associations at one particular point in time. A longitudinal design where the outcomes and exposures are measured and monitored repeatedly may produce more accurate results.

6. Conclusion

This study has provided evidence that the XMEs, in particular PON1-192, NAT2 and GSTM1 may modify OP neurotoxicity. In order to identify susceptible populations and determine whether preventative strategies can be developed for the vulnerable groups, larger longitudinal studies and repeated pesticide bio-monitoring are needed for these gene-exposure interaction studies.

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SUPPLEMENTARY TABLES TO JOURNAL MANUSCRIPT

PART D: APPENDICES

APPENDIX A: Exposure and Outcome Questionnaires

(ENGLISH)

Neurobehavioural effects of pesticide exposure among emergent farmers in the Western Cape

Farmer Questionnaire

UNIVERSITY OF CAPE TOWN

Questionnaire Number	
District	
Date	
Farm (Trust/project) name	
Name of Farmer	
Cell phone number	
Telephone number (landline)	
Name of Interviewer	

GENERAL INSTRUCTIONS

Thank you for agreeing to take part in this study.

We will work through the questionnaire as follows: I will ask the questions and give you the answer choices and tick or circle the answers you give me in the questionnaire. Choose the answer that is the closest to how you feel. The interview will take between forty five minutes and one hour to complete.

Please note that there are no right or wrong answers to the questions asked. Please feel free to answer just what you think. You may stop at any time if you do not want to carry on with these questions. Your answers are confidential and will not be shared with anyone. Only the research staff will have access to the questionnaire once it has been completed.

Section 1: DEMOGRAPHIC CHARACTERISTICS

We would like to ask you a few questions about yourself.

Please circle the correct response.

1.1 Gender Male / Female

1.2 How old are you? _____years

Date of birth ____/_____/_____

1.3 What is the highest level of education you have passed?

Less than one year completed	1
Sub A/Class 1/Grade 1	2
Sub B/Class 2/Grade 2	3
Standard 1/Grade 3	4
Standard 2/Grade 4	5
Standard 3/Grade 5	6
Standard 4/Grade 6	7
Standard 5/Grade 7	8
Standard 6/Grade 8	9
Standard 7/Grade 9	10
Standard 8/Grade 10	11
Standard 9/Grade 11	12
Standard 10/Grade 12	13
Further studies – incomplete	14
Diploma/other post school – complete	15
Degree	16

1.4 Which of the following is the main language spoken at home? (Please circle only one)

English	1
Afrikaans	2
IsiXhosa	3
IsiZulu	4
SeSotho	5
SeTswana	6
SePedi	7
SiSwati	8
TshiVenda	9
Zitsonga	10
IsiNdebele	11

Other (Please specify)	12

Section 2: HOUSEHOLD FACTORS

2.1 Is the house you live in:

Owned by your family	1
Rented	2
Owned by the owner of the farm	3
Other (please specify)	4

2.2 How many rooms are there in this house?

	Rooms

2.3 How many bedrooms are there in this house?

	Bedrooms

2.4 How many bathrooms are there in this house?

	Bathrooms

2.5 Does your house have:

		Yes	No
A	Electricity		
B	A radio		
C	A television		
D	A landline telephone		
E	A fridge		
F	A computer		
G	A washing machine		
H	A cell phone (anybody)		

2.6 Which of the following live in the same household with you?

		Yes	No
A	Live alone		
B	Partner		
C	Child or Children under 13 yrs		
D	Child or Children over 13 yrs		
E	Brother(s) and/or sister(s)		

F	Mother/Female guardian		
G	Father/Male guardian		
H	Grandparent(s)		
I	Other (please specify)		

2.7 How many people usually live and sleep in your household?

	Number of people

Section 3: ECONOMIC FACTORS

Now we would like to ask a few questions about you and the work that you do.

3.1 What kind of work do you do? (If working, please tell me your occupation. For example, Farmer, Street Trader, Primary School Teacher, Domestic Worker)

Not working	0
Working (Please specify)	1

3.2 Have you done any paid work in the last 12 months?

No	0
Yes	1

3.3 What kind of paid work did you?

1.	
2.	
3.	

3.4 Please indicate which of the following are your sources of income. Please answer this question whether or not you are working.

		Yes	No
A	Work		
B	Spouse/partner		
C	Parents		
D	Brothers and/or sisters		
E	Children		
F	Child Support Grant		
G	State Old Age Pensions		

H	Disability Grant		
I	Care Dependency Grant		
J	Foster Care Grant		
K	Grants-in-Aid		
L	Workman's Compensation Fund		
M	Other (Please specify)		

3.5 How often do the people here go hungry or have no food to eat?

Never	0
Seldom	1
Sometimes	2
Often	3

3.6 How often does your family have enough money for:

		Never	Some-times	Always	Not Applicable
A	Buying food	0	1	2	3
B	Paying for transport (bus, taxi, train fare, petrol bills)	0	1	2	3
C	Paying bills (rent, light, water, telephone, etc.)	0	1	2	3
D	Paying doctors and for medicine	0	1	2	3
E	Buying school supplies, uniforms, books, shoes	0	1	2	3
F	Buying clothes	0	1	2	3
G	Buying firewood, coal, paraffin	0	1	2	3

Section 4. LIFE HISTORY

Now I'd like to ask you a few questions about the places where you have lived in your lifetime:

4.1 Where do you live now ? _____

4.2 How long have you lived here? _____ _(Years/Months)

4.3 What kind of farm is this (what is grown here) ? _____

4.4 How far from your house is the vineyard/field ? _____

4.5 Are pesticides sprayed on the vineyard/field during the year? (YES, NO)

IF YES, complete the following:

4.5.1 How many times a year are pesticides applied by means of a tractor with a boom sprayer _____ (number of times a year)

4.5.2 A tractor with persons using hand or backpacks? _____ (number of times a year)

4.5.3 Aerial spraying (with an aeroplane) _____ (number of times a year)

4.5.4 Quadbike spraying _____(number of times a year)

4.6 Does the pesticide spraying come into the house? (YES, NO)

4.7 Does any member of your family come into contact with pesticides outside the house while spraying occurs (eg. children playing near spraying area) ? (YES, NO)

4.8 Does any member of your family go into in the field/vineyards soon after spraying or come into contact with sprayed surfaces? (YES, NO)

4.9 What are the sources of drinking water at your house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

4.10 What are the sources of water for recreational use (bathing, washing of clothes) at your house? _____ (municipal water, storage dam on mountain, borehole/spring, river water, farm dam, rain water tank, etc)

4.11 Does any other member of your family perform work on the farm? (YES, NO)

If Yes – list the members who are involved in spraying and/or mixing pesticides? (for example – partner/wife – mixes pesticides or adult member of the family sprays pesticides)

Member of family e.g. adult son	Activity e.g. sprays pesticides

4.12 Do you work in the pesticide store? (YES, NO)

4.13 Does any member of your family come into contact with empty pesticide containers? (YES, NO)

If YES, how _____ (for example drinking water, burn empty containers)

4.14 Does any member of your family eat from the crops in the vineyard/field soon after spraying? (YES, NO)

4.15 Do you wash your hands before you eat? (YES, NO)

Section 5. ALCOHOL USE

5. Do you drink alcohol ? (YES, NO)

5.1 Have you ever felt that you should drink less alcohol? (YES, NO)

5.2 Have people ever angered you by criticising your drinking habits? (YES, NO)

5.3 Have you ever felt guilty or bad because you drink alcohol? (YES, NO)

5.4 Have you ever had a drink early in the morning to make you feel better or to get over a 'babalaas'? (YES, NO)

Section 6. SMOKING AND OTHER DRUG USE

6.1 Do you smoke ? (YES, NO)

6.1.1 **If YES** please state what you smoke:

	YES	NO
Cigarettes		
Pipe Tobacco		
Dagga		
Other, please describe		

6.1.2 **If NO** did you ever smoke and if so what did you smoke? (YES, NO)
(IF you did smoke before please state what you smoked)

	YES	NO
Cigarettes		

Pipe Tobacco		
Dagga		
Other, please describe		

6.2 During the past 30 days, on how many days did you use each of the following substances if at all?

		0 days	1 or 2 days	3 to 5 days	6 to 9 days	10 to 19 days	20 to 29 days	All 30 days
A	Dagga	0	1	2	3	4	5	6
B	Mandrax	0	1	2	3	4	5	6
C	Heroin	0	1	2	3	4	5	6
D	Crack/cocaine	0	1	2	3	4	5	6
E	Ecstasy	0	1	2	3	4	5	6
F	Methamphetamine (tik)	0	1	2	3	4	5	6
I	Other	0	1	2	3	4	5	6

Section 7. SUICIDALITY

7.1 During the past 12 months have you ever seriously thought about hurting yourself in a manner that may cause you to die? (YES, NO)

7.2 During the past 12 months, have you ever told someone that you plan to commit suicide? (YES, NO)

7.3 During the past 12 months, have you ever tried to commit suicide? (YES, NO)

7.4 Have any of your attempts to injure yourself caused you to be treated by a doctor or nurse? (YES, NO)

Section 8. WORK HISTORY

Current job

8.1 Are you currently working on this farm (YES/NO)

8.1.1 If YES - how long have you been working on this farm (or since when)

(If NO – Go to question 8.2)

_____

8.1.2 Which crops do you work with _____

8.1.3 Do you apply pesticides (YES/NO)

8.1.3.1 If YES which pesticides do you use _____

_____

Please provide the details about your **CURRENT** work with pesticides in the following table

Current job – Date: _____

Activity	Yes/No	Hours per day And Days per week	Weeks per month And Months per year	Number of years	PPE Use: Indicate which: A = Apron B = Boots G = Gloves M = Mask O = Overalls Gls = Goggles
Mix pesticides inside					
Mix pesticides outside					
Tractor driver with boom sprayer					
Tractor driver without boom sprayer					
Quadbike spraying					
Back pack or hand spraying					

8.2 If NO – please answer the following questions:

8.2.1 Are you currently also doing a non-farming job (YES/NO)

8.2.2 If yes, where are you working _____

8.2.3 What is your job title _____

8.3 Please list your **PREVIOUS jobs** for the last 3 years (excluding your current job) where you handled, mixed or in any way came into contact with pesticides. Start with the job before your current job.

JOB 1 - Date:_____

Activity	Yes/No	Hours per day And Days per week	Weeks per month And Months per year	Number of years	PPE Use: Indicate which: A = Apron B = Boots G = Gloves M = Mask O = Overalls Gls = Goggles
Mix pesticides inside room					
Mix pesticides outside room					
Tractor driver with boom sprayer					
Tractor driver without boom sprayer					
Quadbike spraying					
Back pack or hand spraying					

JOB 2 - Date:_____

Activity	Yes/No	Number of months per year	Number of days per week	Number of years	PPE Use: Indicate which: A = Apron B = Boots G = Gloves M = Mask O = Overalls Gls = Goggles
Mix pesticides inside room					
Mix pesticides outside room					
Tractor driver with boom sprayer					

Tractor driver without boom sprayer					
Quadbike spraying					
Back pack or hand spraying					

JOB 3 - Date:_____

Activity	Yes/No	Hours per day And Days per week	Weeks per month And Months per year	Number of years	PPE Use: Indicate which: A = Apron B = Boots G = Gloves M = Mask O = Overalls Gls = Goggles
Mix pesticides inside room					
Mix pesticides outside room					
Tractor driver with boom sprayer					
Tractor driver without boom sprayer					
Quadbike spraying					
Back pack or hand spraying					

Section 9. ENVIRONMENTAL EXPOSURE

9.1 Do you use any pesticides in your garden or in your home? (YES / NO)
eg. Target or Doom _____

9.2 For how long have you been using pesticides at home _____ (number of years)

9.3 How frequently do you use pesticides at home _____ (every day, 3 times a week, once a week, once a month, less than once a month)

9.4 Have you taken any empty containers home? (YES / NO)

9.5 If yes, what was it used for _____

9.6 Does any other person in the house work with pesticides? (YES / NO)

9.7 If yes, how many? _____

9.8 For how long has this person in your home worked with pesticides? _____

_____(Year(s))

9.9 Do pesticide contaminated clothes get washed at home (YES / NO)

9.10 If yes, does it get washed with the rest of the washing? (YES / NO)

9.11 Do you eat fruit or vegetables from your garden (YES / NO)

9.12 Do you use empty pesticide containers at home for domestic purposes (YES / NO)

9.13 If yes, what do you use them for? _____

_____

9.14 For how long have you been using empty containers at home _____ (Year(s))

9.15 What do you do with the pesticide containers you don't use anymore

_____

Brief Symptom Inventory (BSI)

(Interviewer write the answer of the worker, according to the given ratings, in the appropriate block)

NOT AT ALL	**0**
A LITTLE BIT	**1**
MODERATELY	**2**
QUITE A BIT	**3**
EXTREMELY	**4**

This is a list of problem that people sometimes experience. Please listen to each one Carefully and choose the one which best describes **THE EXTENT TO WHICH THIS PROBLEM HAS UPSET/DISTURBED YOU DURING THE PAST 7 DAYS, INCLUDING TODAY.**

1. Nervousness or shakiness inside	
2. Faintness or dizziness	
3. The idea that someone else can control your thoughts	
4. Feeling others are to blame for most of your troubles	
5. Trouble remembering things	
6. Feeling easily annoyed or irritated	
7. Pains in heart or chest	
8. Feeling afraid in open spaces or on street	
9. Thoughts of ending your life	
10. Feeling that most people cannot be trusted	
11. Poor appetite	
12. Suddenly scared for no reason	
13. Temper outbursts that you could not control	
14. Feeling lonely even when you are with people	
15. Feeling blocked in getting things done	
16. Feeling lonely	
17. Feeling blue	
18. Feeling no interest in anything	
19. Feeling fearful	
20. Your feelings being easily hurt	
21. Feeling that people are unfriendly or dislike you	
22. Feeling inferior to others	
23. Nausea or upset stomach	
24. Feeling that you are watched or talked about by others	
25. Trouble falling asleep	
26. Having to check and double-check what you do	
27. Difficulty making decisions	
28. Feeling afraid to travel on buses, subways or trains	
29. Trouble getting your breath	
30. Hot or cold spells	

31. Having to avoid certain things, places or activities because they frighten you	
32. Your mind going blank	
33. Numbness or tingling in parts of your body	
34. The idea that you should be punished for your sins	
35. Feeling hopeless about the future	
36 Trouble concentrating	
37. Feeling weak in parts of your body	
38. Feeling tense or keyed up	
39. Thoughts of death or dying	
40. Having urges to beat, injure or harm someone	
41. Having urges to break or smash things	
42. Feeling very self-conscious with others	
43. Feeling uneasy in crowds such as shopping or at a movie	
44. Never feeling close to another person	
45. Spells of terror or panic	
46. Getting into frequent arguments	
47. Feeling nervous when you are left alone	
48. Others not giving you proper credit for your achievements	
49. Feeling so restless you couldn't sit still	
50. Feelings of worthlessness	
51. Feeling that people will take advantage of you if you let them	
52. Feelings of guilt	
53. The idea that something is wrong with your mind	

Q16 SYMPTOMS

Please circle the correct answer

1. Are you abnormally tired ? 1. YES 2. NO

2. Do you have palpitations of the heart when you do not exert yourself ? 1. YES 2. NO

3. Do you often have painful tingling in some part of your body ? 1. YES 2. NO

4. Do you often feel irritated without any particular reason ? 1. YES 2. NO

5. Do you often feel depressed without any particular reason ? 1. YES 2. NO

6. Do you often have problems concentrating ? 1. YES 2. NO

7. Do you have a short memory ? 1. YES 2. NO

8. Do you often perspire without any particular reason ? 1. YES 2. NO

9. Do you have any problems with buttoning and unbuttoning ? 1. YES 2. NO

10.Do you generally find it hard to get the meaning from reading newspapers and books ? 0. standard 4 or less = CANNOT READ 1. YES 2. NO

11.Have your relatives told you that you have a short memory ? 1. YES 2. NO

12. Do you sometimes feel a heavy feeling on your chest ? 1. YES 2. NO

13. Do you often have to make notes about what you must remember ? 0. standard 4 or less = CANNOT READ 1. YES 2. NO

14. Do you often have to go back and check things you have done such as locking the door ? 1. YES 2. NO

15. Do you have a headache at least once a week ? 1. YES 2. NO

16. How many times do you have sex per week?_____

16a. Do you think that this is less than most persons of your age? 1. YES 2. NO

Thank you for taking part in this study

APPENDIX B: STUDY INFORMATION SHEETS

(ENGLISH)

STUDY INFORMATION SHEET

Adult Family member

1. Title of research project

The title of the study is "neurobehavioural effects of organophosphate pesticides among emergent farmers and adult members of their families in the Western Cape."

2. Purpose of research

The purpose of this research is to find out whether you and adult members of your family have been affected negatively because of coming into close contact with, and/or using pesticides. In particular the study will look at the effects of pesticides that we call organophosphates on your health as well as the health of adult members of your family.

3. Why the research is important?

South African farmers, farm workers and their families may be affected by pesticides. Emerging farmers or small-scale farmers may be encouraged to use more pesticides in order to become commercial farmers. Without the necessary information and training about the effects and use of pesticides the health of farmers and members of their family may be at risk. Because the effects of using low doses of pesticides over a long period of time are not always visible and noticeable farmers and their families may not be aware of the dangers that these pesticides may cause to their health.

4. Description of the research project

The study will take place over twelve months starting approximately December 2008 in the following manner:
At the start of the study, we will do the following: a) ask you to answer question from a questionnaire; b) perform some tests of your memory, your thinking and your mood, and your movements and administer questionnaires. These visits will take place approximately in March, June, September with the last testing taking place in December 2009.

If you agree to participate, and if you give permission for adult members of your family to take part in the study, I and four research assistants will administer the following tests to each adult:

- A questionnaire that will take between 30 and 45 minutes each to complete. The questionnaire that will ask questions about your age, gender, education, health, drinking, smoking practices and work activities, your psychological well-being and your general health.
- Tests of your memory, your thinking your mood, and your movements. These are like IQ tests and will not cause any harm to you or your family. These are tests that will involve you telling me things, pointing out things and moving your hands.
- An examiner will test how well you can feel vibration in your ankle using a tuning fork

Whenever possible, you will be interviewed in privacy in order to complete the questionnaire and to conduct the tests. Any personal information will be kept confidential.

5. Risks and discomforts of the research

There are no risks when completing the questionnaires. However testing will be stopped if you as the participant request it and can be continued at a later time or not at all. If you are uncomfortable about any aspect of the testing, intervention or treatment will be made available from a trained clinical staff member.

6. Expected benefits to you and others
This study will try to find out whether you have been affected negatively because of using and/or coming into close contact with pesticides in your farming activities and if you have been affected, you will be provided with information about pesticides and referred to your nearest hospital or clinic if necessary. It is important that you and adult members of your family attend all of these testing sessions to determine the effects of pesticide exposure on your health.

7. Costs to you resulting from participation in the study
You will not be paid or have to pay for taking part in the study. You will be provided with lunch or tea if you have to spend a long time with us in the study.

8. Confidentiality of information collected
You will not be personally identified in any reports on this study. The records will be kept confidential to the extent provided by law.

9. Documentation of the consent
A copy of this document will be kept together with my research records on this study.

10. Contact person
You may contact the following persons for answers to further questions about the research, your rights, or any injury you may feel relates to the study.

Professor Marc Blochman (Chairperson of the Research Ethics Committee)
Telephone: 021 406 6492

Name of researcher: Ms. Zelda Holtman (Student and researcher)
Telephone: 021 406 6842
Fax: 021 406 6163
Email: Zelda.Holtman@uct.ac.za

Name of Principal Co-investigator: Dr Aqiel Dalvie
Telephone: 021 406 6610
Fax: 021 406 6163
Email: Aqiel.Dalvie@uct.ac.za

11. Voluntary nature of participation
Your participation in this project is entirely voluntary. You must decide whether you want to participate or not, without feeling obligated to anyone. You will not suffer any discrimination from the extension officers or the health services if you decide you do not want to participate. Even if you start participating in the study, you can always change your mind later and withdraw from the study.

APPENDIX C: CONSENT FORMS

(ENGLISH)

Consent Form

Head of Household

Consent to participate in study on the neurobehavioural effects of Organophosphate pesticides among emergent farmers and adult members of their families.

I am a PhD student at the University of Cape Town. I would like to ask you to participate in a research study.

1. Title of research project

The title of the study is "The neurobehavioural effects of organophosphate pesticides among emergent farmers and adult members of their families in the Western Cape."

2. Purpose of research

The purpose of this research is to find out whether you and adult members of your family have been affected negatively because of coming into close contact with, and/or using pesticides. In particular the study will look at the effects of pesticides that we call organophosphates on your health as well as the health of adult members of your family.

3. **Why the research is important?**
South African farmers, farm workers and their families may be affected by pesticides. Emerging farmers or small-scale farmers may be encouraged to use more pesticides in order to become commercial farmers. Without the necessary information and training about the effects and use of pesticides the health of farmers and members of their family may be at risk. Because the effects of using low doses of pesticides over a long period of time are not always visible and noticeable farmers and their families may not be aware of the dangers that these pesticides may cause to their health.

4. Description of the research project
The study will take place over twelve months starting approximately December 2008 in the following manner:
At the start of the study, we will do the following: a) ask you to answer question from a questionnaire; b) perform some tests of your memory, your thinking and your mood. After this initial examination, we will visit you and the adult members of your family who have consented to participate every third to fourth month to administer questionnaires. These visits will take place approximately in March, June, September with the last testing taking place in December 2009

If you agree to participate, and if you give permission for adult members of your family to take part in the study, I and four research assistants will administer the following tests to each adult:

- A questionnaire that will take between 30 and 45 minutes each to complete. The questionnaire that will ask questions about your age, gender, education, health, drinking, smoking practices and work activities, your psychological well-being and your general health.
- Tests of your memory, your thinking your mood, and your movements. These are like IQ tests and will not cause any harm to you or your family. These are tests that will involve you telling me things, pointing out things and moving your hands.
- An examiner will test how well you can feel vibration in your ankle using a Tuning Fork
- A checklist that will record the number, type and severity of all injuries

Whenever possible, you will be interviewed in privacy in order to complete the questionnaire and to conduct the tests. Any personal information will be kept confidential.

5. Risks and discomforts of the research
There are no risks when completing the questionnaires. However testing will be stopped if you as the participant request it and can be continued at a later time or not at all. If you are uncomfortable about any aspect of the testing, intervention or treatment will be made available from a trained clinical staff member.

6. Expected benefits to you and others

This study will try to find out whether you have been affected negatively because of using and/or coming into close contact with pesticides in your farming activities and if you have been affected, you will be provided with information about pesticides and referred to your nearest hospital or clinic if necessary. It is important that you and adult members of your family attend all of these testing sessions to determine the effects of pesticide exposure on your health.

7. Costs to you resulting from participation in the study
You will not be paid or have to pay for taking part in the study. You will be provided with lunch or tea if you have to spend a long time with us in the study.

8. Confidentiality of information collected
You will not be personally identified in any reports on this study. The records will be kept confidential to the extent provided by law.

9. Documentation of the consent
A copy of this document will be kept together with my research records on this study.

10. Contact person
You may contact the following persons for answers to further questions about the research, your rights, or any injury you may feel relates to the study.

Professor Marc Blochman (Chairperson of the Research Ethics Committee)
Telephone: 021 406 6492

Name of researcher: Ms. Zelda Holtman (Student and researcher)
Telephone: 021 406 6842
Fax: 021 406 6163
Email: Zelda.Holtman@uct.ac.za

Name of Principal Investigator: Professor Leslie London
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Email: Leslie.London@uct.ac.za

Name of Principal Co-investigator: Dr Aqiel Dalvie
Telephone: 021 406 6610
Fax: 021 406 6163
Email: Aqiel.Dalvie@uct.ac.za

11. Voluntary nature of participation
Your participation in this project is entirely voluntary. You must decide whether you want to participate or not, without feeling obligated to anyone. You will not suffer any discrimination from the extension officers or the health services if you decide you do not want to participate. Even if you start participating in the study, you can always change your mind later and withdraw from the study.

Consent of the participant

Head of household
I have read the information given above. I understand the meaning of this information. I, Mr./Ms/Mrs _____have offered to answer any questions concerning the study. I hereby consent to participate in the study.

I understand that an adult member of my family will also be asked to participate in the study and I Mr./Ms/Mrs _____ as head of my household hereby give my consent for my wife/partner/adult daughter/adult son to be approached to participate in the study.

_____	_____	
Printed name of participant (Head of household)	**signature**	**Date**
_____	_____	
Interviewer (print)	**signature**	**Date**
_____	_____	
Printed name of witness (if participant is unable to read and write)	**signature**	**Date**

APPENDIX D: Supplementary Tables to Journal Manuscript

Table 9: Description of exposure information

Variables		N (%)		Median (IQR)
Current Job				
Farm worker	Total		283 (94)	
	Pesticide Applicator	160 (53.2)		
	General Worker	6 (2.0)		
	Infield Non-applicator	117 (38.9)		
Non-farm worker	Total		18 (6)	
	Industry	1 (0.3)		
	Other	9 (3.0)		
	Pensioner	6 (2.0)		
	Unemployed	2 (0.7)		
Pesticide Exposure	Number of job years			8 (4-14)
	Pesticide exp.	157 (52.3)		
	No Pesticide exp.	143 (47.8)		
Applicator status				
Past and current Applicators (All pesticides)	Non-applicators	141 (46.8)		
	Applicators	160 (53.2)		
Current Applicators (All pesticides)	Non-applicators	152 (50.7)		
	Applicators	148 (49.3)		
OP Applicators	Non-OP Applicator	234 (77.7)		
	OP Applicator	67 (22.3)		
Past poisoning				
Diagnosed poisoned cases		22 (11.5)		
Non-cases		170 (88.5)		
All pesticide exposure				
Long term exposure days (8 hr days)				543.8 (71.1-1460.4)
Jem days weighted by activity				369.3 (52.3-1022.3)
Jem days weighted by activity & crop type				4182.6 (544.8-144452.1)
Jem days weighted by activity & crop type per annum				428.0 (83.8-1348.7)
OP pesticide exposure				
Long term exposure days (8 hr days)				308.0 (45.0-953.4)
Jem days weighted by activity				215. 6 (31.5-667.4)
Jem days weighted by activity & crop type				1950.9 (339.0-7545.9)
Jem days weighted by activity & crop type per annum				143.3 (36.1-732.1)
Type of crop farming				
Any Combination		66 (21.9)		
Citrus		68 (22.6)		
Deciduous Fruit		79 (26.3)		

Variables	N (%)		Median (IQR)
Grapes	20 (6.6)		
Vegetables	50 (16.6)		
Other Crops	13 (4.3)		
No crops	5 (1.6)		

Table 10:Demographic,Llifestyle and Socioeconomic factors by the different Neurobehavioural Outcomes

		Digit Span Forward		Digit Span Backward		Digit Span WAIS		Vibration Sensitivity	
		N	Median	N	Median	N	Median	N	Median
Demographic Variables									
Gender	Female	97	6 (5-7)	97	4 (3-4)	97	7 (6-8)	97	12.50 (9-15)
	Male	204	6 (4.50-7)	204	3 (2-4)	204	6 (6-8)	204	13.50 (10-17.25)
Language	Afrikaans	262	6 (5-7)	262	3 (2-4)	262	6 (6-8)	259	12.50 (9-17)
	Xhosa	31	5 (4-7)	31	3 (2-4)	31	6 (5-8)	30	14.50 (12.00-16.50)
	Sotho	8	5.5 (4-7)	8	3.5 (3-6)	8	7 (5-9)	8	13.25 (12.25-14.5)
Education	≤9 years education	197	6 (4-7)	197	3 (2-4)	197	6 (5-8)	194	13 (9.50-16.50)
	>9 years education	104	6 (5-8)	104	4 (3-5)	104	7 (6-9)	103	13 (10-17)
District	Overberg	69	6 (5-7)	69	3 (2-4)	69	6 (6-8)	69	13 (9.50-16)
	Winelands	148	6 (5-7.50)	148	4 (3-4)	148	6 (6-8)	145	12.50 (9-16.50)
	West Coast	84	6 (4-8)	84	3 (2-4)	84	6 (5-8)	83	14 (10-17.50)
Crop code	Any Combination	66	6 (5-8)	66	4 (3-4)	66	7 (6-8)	64	13 (8.50-17.75)
	Citrus Fruits	68	6 (4-8)	68	3 (2-4)	68	6 (5-8)	67	14 (10-17)
	Deciduous Fruits	79	6 (4-7)	79	3 (2-4)	79	6 (6-8)	79	12 (8-16)
	Grapes	20	5 (5-7)	20	4 (2.5-4)	20	6 (6-7.5)	20	12.25 (9.25-15.75)
	No crops	5	5 (3-6)	5	3 (3-3)	5	6 (5-6)	5	13 (9-14)
	Other	13	7 (5-10)	13	4 (4-5)	13	8 (7-11)	13	16 (11.5-17)
	Vegetables	50	6 (5-8)	50	4 (2-4)	50	7 (6-8)	49	13 (10-16.5)
Lifestyle Factors									
CAGE Score	Not Problem alcohol users (≤2)	162	6 (5-7)	162	4 (2-4)	162	7 (6-8)	159	13 (9.5-16.5)
	Problem alcohol users (≥2)	139	6 (5-7)	139	3 (2-4)	139	6 (5-8)	138	12.25 (9-17)
Current Alcohol Consumption	YES	188	6 (5-7)	188	3 (2-4)	188	6 (6-8)	186	12.5 (9.5-16.5)
	NO	113	6 (5-7)	113	4 (2-4)	113	7 (6-8)	111	13.5 (9.5-17)
Smoking Habits	Non-current Smoker	100	6 (5-7)	100	3.5 (2-4)	100	6.5 (6-8)	100	12 (9.5-16)
	Current Smoker	201	6 (5-7)	201	3 (2-4)	201	6 (5-8)	197	13.5 (9-17)
Previous Head Injuries	Reported Head Injury	35	7 (5-8)	35	3 (3-4)	35	7 (6-8)	33	15.5 (12-20)
	No-reported Injury	266	6 (5-7)	266	3 (2-4)	266	6 (6-8)	264	12.5 (9.25-16.5)
	Reported Illness	5	5 (5-5)	5	4 (3-4)	5	6 (5-6)	5	17 (14.5-20)

		Digit Span Forward		Digit Span Backward		Digit Span WAIS		Vibration Sensitivity	
		N	Median	N	Median	N	Median	N	Median
Psychiatric Illness	Non-reported Illness	296	6 (5-7)	296	3 (2-4)	296	6 (6-8)	292	13 (9.5-16.5)
Socioeconomic Status									
Owns >3 items		264	6 (5-8)	264	3 (2-4)	264	6 (6-8)	261	13 (9-16.5)
Owns ≤3 items		37	6 (5-8)	37	4 (2-4)	37	6 (6-8)	36	14.5 (11.5-17.75)
No Grant		201	6 (5-8)	201	4 (2-4)	201	6 (6-8)	198	13 (9.5-16.5)
Grant		100	6 (5-7)	100	3 (2-4)	100	6 (6-8)	99	12.5 (9.5-17)
Not Hungry		258	6 (5-8)	258	3 (2-4)	258	6 (6-8)	255	13 (9.5-17)
Hungry occasionally		43	6 (5-7)	43	4 (2-5)	43	7 (5-8)	42	13.5 (9.5-16.5)

Table 11: Demographic, Lifestyle and Socioeconomic Information by Genetic Polymorphisms

		GSTT		GSTM		NAT			PON1_55			PON1_192		
		NULL	YES	NULL	YES	GG	GA	AA	TT	TA	AA	GG	GA/AG	AA
Demographic Variables														
Gender	F	3 (3.09)	94 (96.91)	21 (21.65)	76 (78.35)	85 (87.63)	11 (11.34)	1 (1.03)	65 (67.01)	30 (30.93)	2 (2.06)	47 (48.45)	37 (38.14)	13 (13.40)
	M	1 (0.49)	203 (99.51)	27 (13.24)	177 (86.76)	187 (91.67)	16 (7.84)	1 (0.49)	144 (70.59)	54 (26.47)	6 (2.94)	91 (44.61)	93 (45.59)	20 (9.80)
Age (years)		46.50 (33.00-50.50)	39.00 (30.00-48.00)	38 (32-44)	40 (30-49)	39 (30-48)	43 (33-53)	39 (39-39)	40 (30-49)	37.50 (30.50-44)	41.50 (30.00-50.00)	38.50 (29.00-46.00)	40 (31-48)	40 (29-500)
Language	A	3 (1.15)	259 (98.85)	40 (15.27)	222 (84.73)	234 (89.31)	26 (9.92)	2 (0.76)	182 (69.47)	73 (27.86)	7 (2.67)	123 (46.95)	110 (41.98)	29 (11.07)
	X	0	31 (100)	6 (19.35)	25 (80.65)	30 (96.77)	1 (3.23)	0	22 (70.97)	8 (25.81)	1 (3.23)	12 (38.71)	16 (51.61)	3 (9.68)
	S	1 (12.50)	7 (87.50)	2 (25)	6 (75)	8 (100)	0	0	5 (62.5)	3 (37.50)	0	3 (37.5)	4 (50)	1 (12.5)
Education (Years)	≤9	1 (0.96)	103 (99.04)	17 (16.35)	87 (83.65)	93 (89.42)	10 (9.62)	1 (0.96)	66 (63.46)	35 (33.65)	3 (2.88)	-5481.00%	37 (35.58)	10 (9.62)
	>9	3 (1.52)	194 (98.48)	31 (15.74)	166 (84.26)	179 (90.86)	17 (8.63)	1 (0.51)	143 (72.59)	49 (24.87)	5 (2.54)	81 (41.12)	93 (47.21)	23 (11.68)
District	O	2 (2.90)	67 (97.10)	15 (21.74)	54 (78.26)	62 (89.86)	7 (10.14)	0	53 (76.81)	15 (21.74)	1 (1.45)	36 (52.17)	24 (34.78)	9 (13.04)
	W	2 (1.35)	146 (98.65)	28 (18.92)	120 (81.08)	128 (86.49)	18 (12.16)	2 (1.35)	86 (58.11)	57 (38.51)	5 (3.38)	64 (43.24)	65 (43.92)	19 (12.84)
	WC	0	84 (100)	5 (5.95)	79 (94.05)	82 (97.62)	2 (2.38)	0	70 (83.33)	12 (14.29)	2 (2.38)	38 (45.24)	41 (48.81)	5 (5.95)
Crop code	Cb	0	66 (100)	14 (21.21)	52 (78.79)	59 (89.39)	6 (9.09)	1 (1.52)	41 (62.12)	24 (36.36)	1 (1.52)	31 (46.97)	28 (42.42)	7 (10.61)
	CF	0	68 (100)	5 (7.35)	63 (92.65)	66 (97.06)	2 (2.94)	0	58 (85.29)	8 (11.76)	2 (2.94)	30 (44.12)	33 (48.53)	5 (7.35)
	DF	1 (1.27)	78 (98.73)	21 (26.58)	58 (73.42)	67 (84.81)	12 (15.19)	0	61 (77.22)	16 (20.25)	2 (2.53)	35 (44.3)	32 (40.51)	12 (15.19)
	G	2 (10)	18 (90)	3 (15)	17 (85)	17 (85)	3 (15)	0	6 (80)	12 (60)	2 (10)	4 (20)	13 (65)	3 (15)
	N	0	5 (100)	1 (20)	4 (80)	5 (100)	0	0	4 (61.54)	1 (20)	0	3 (60)	2 (40)	0
	O	0	13 (100)	0	13 (100)	12 (92.31)	1 (7.69)	0	8 (61.54)	5 (38.46)	0	6 (46.15)	6 (46.15)	1 (7.69)
	V	1 (2)	49 (98)	4 (8)	46 (92)	46 (92)	3 (6)	1 (2)	31 (62)	18 (36)	1 (2)	29 (58)	16 (32)	5 (10)
Height		1.57 (1.52-1.63)	1.64 (1.58-1.71)	1.64 (1.57-1.70)	1.64 (1.58-1.71)	1.64 (1.58-1.71)	1.61 (1.56-1.68)	1.55 (1.53 - 1.57)	1.63 (1.58-1.71)	1.64 (1.57-1.71)	1.68 (1.64-1.69)	1.63 (1.57-1.71)	1.64 (1.59-1.71)	1.64 (1.57-1.68)
Weight		73.00 (61.00-82.50)	62.00 (55.00-73.00)	65.00 (58.00-77.50)	62.00 (55.00-71.00)	63 (56-74)	60.00 (54.00-71)	61 (42-80)	61.50 (55.00-75.00)	63.00 (55.00-70.00)	58.50 (53.00-62.50)	64 (56-75)	61 (55-70)	60 (51-70)
BMI		31.55 (25.00-33.44)	22.77 (20.05-27.36)	23.78 (20.42-30.09)	22.77 (19.79-27.06)	22.83 (20.05-27.46)	22.43 (21.22-25.39)	25.61 (17.04-34.17)	22.87 (20.07-27.40)	22.68 (19.94-28.25)	21.13 (18.78-23.36)	22.96 (20.76-28.37)	22.55 (19.66-26.67)	22.04 (18.59-26.35)
Lifestyle Factors														

		GSTT		GSTM		NAT			PON1_55			PON1_192		
		NULL	YES	NULL	YES	GG	GA	AA	TT	TA	AA	GG	GA/AG	AA
CAGE Score	No case	3 (1.85)	159 (98.15)	27 (16.67)	135 (83.33)	147 (90.74)	15 (9.26)	0	106 (65.43)	54 (33.33)	2 (1.23)	74 (45.68)	73 (45.06)	15 (9.26)
	case	1 (0.71)	138 (99.28)	21 (15.11)	118 (84.89)	125 (89.93)	12 (8.63)	2 (1.44)	103 (74.10)	30 (21.58)	6 (4.32)	64 (46.04)	57 (41.01)	18 (12.95)
Current Alcohol Consumption	Yes	2 (1.06)	186 (98.94)	35 (18.62)	153 (81.38)	169 (89.98)	17 (9.04)	2 (1.06)	132 (70.21)	50 (26.60)	6 (3.19)	86 (45.74)	79 (42.02)	23 (12.23)
	No	2 (1.77)	111 (98.23)	13 (11.50)	100 (88.50)	103 (91.15)	10 (8.85)	0	77 (68.14)	34 (30.09)	2 (1.77)	52 (46.02)	51 (45.13)	10 (8.85)
Current smoking Habits	Non	3 (3)	97 (97)	14 (14)	86 (86)	88 (88)	12 912)	0	70 (70)	28 (18)	2 (2)	46 (46)	45 (45)	9 (9)
	Yes	1 (0.5)	200 (99.5)	34 (16.92)	167 (83.08)	184 (91.54)	15 (7.46)	2 (1)	139 (69.15)	56 (27.86)	6 (2.99)	92 (45.77)	85 (42.29)	24 (11.94)
Previous Head Injuries	Yes	0	35 (100)	3 (8.57)	32 (91.43)	33 (94.29)	2 (5.71)	0	28 (80)	6 (17.14)	1 (2.86)	13 (37.14)	20 (57.14)	2 (5.71)
	No	4 (1.50)	262 (98.5)	45 (16.92)	221 (83.08)	239 (89.85)	25 (9.4)	2 (0.75)	181 (68.05)	78 (29.32)	7 (2.63)	125 (46.99)	110 (41.25)	31 (11.65)
Psychiatric Illness	Yes	0	5 (100)	1 (20)	4 (80)	4 (80)	1 (20)	0	5 (100)	0	0	4 (80)	0	1 (20)
	No	4 (1.35)	292 (98.65)	47 (15.88)	249 (84.12)	268 (90.54)	26 (8.78)	2 (0.68)	204 (68.92)	84 (28.38)	8 (2.7)	134 (45.27)	130 (43.92)	32 (10.81)
Socioeconomic Status														
Owns >3 items		2 (0.76)	262 (99.24)	41 (15.53)	223 (84.47)	240 (90.91)	22 (8.33)	2 (0.76)	181 (68.56)	77 (29.17)	6 (2.27)	120 (45.45)	115 (43.56)	29 (10.98)
Owns ≤3 items		2 (5.41)	35 (94.59)	7 (18.92)	30 (81.08)	32 (86.49)	5 (13.51)	0	28 (75.68)	7 (18.92)	2 (5.41)	18 (48.65)	15 (40.54)	4 (10.81)
No Grant		2 (1)	199 (99)	30 (14.93)	171 (85.07)	180 (89.55)	19 (9.45)	2 (1)	139 (69.15)	55 (27.36)	7 (3.48)	92 (45.77)	86 (42.79)	23 (11.44)
Grant		2 (2)	98 (98)	18 (18)	82 (82)	92 (92)	8 (8)	0	70 (70)	29 (29)	1 (1)	46 (46)	44 (44)	10 (10)
Not Hungry		3 (1.16)	255 (98.84)	40 (15.50)	218 (84.50)	231 (89.53)	25 (9.69)	2 (0.78)	178 (68.99)	72 (27.91)	8 (3.10)	116 (44.96)	116 (44.96)	26 (10.08)
Hungry occasionally		1 (2.33)	42 (97.67)	8 (18.60)	35 (84.05)	41 (95.35)	2 (4.65)	0	31 (72.09)	12 (27.91)	0	22 (51.16)	14 (32.56)	7 (16.28)

APPENDIX E: Instructions to Authors

1. INSTRUCTIONS TO AUTHORS

2. WHO WE ARE

Environmental Health Perspectives (*EHP*) is a monthly open-access journal that publishes peer-reviewed research and news concerning human health and the environment. One of the overarching principles of the journal is to provide a forum for the objective and balanced presentation of scientifically credible information. Although *EHP* is sponsored by the National Institute of Environmental Health Sciences (NIEHS), its editorial policies are independent of the institute.

In 2004 *EHP* became an open-access journal. All content published since the beginning of the journal in 1972 is available free online at http://www.ehponline.org/ andhttp://www.ncbi.nlm.nih.gov/pmc/journals/253/. *EHP* is committed to promoting the discussion and exchange of information internationally, as described in detail athttp://www.ehponline.org/international/.

3. WHAT WE PUBLISH

The environmental health sciences include many fields of study and increasingly comprise multi-disciplinary research areas. *EHP* publishes articles from a wide range of scientific disciplines encompassing mechanistic research, experimental and observational human studies, and *in vitro* and *in vivo* animal research with a clear relationship to human health effects. Studies

involving exposure science, climate change, ecologic issues, or effects on wildlife populations are welcome, but the relevance of the findings to human health should be made clear. *EHP*also addresses ethical, legal, social, and policy issues related to environmental public health. Because children are uniquely sensitive to their environments, *EHP* devotes a research section specifically to issues surrounding children's environmental health.

EHP provides additional information on environmental health issues through its News and Editorials. Although *EHP* welcomes ideas for News and Editorials, the journal does not accept unsolicited manuscripts of these types. Please contact the Editor-in-Chief for further information.

4. ABOUT YOUR MANUSCRIPT

All papers submitted to *EHP* are evaluated by a group of consulting editors to determine whether the topic is within the scope of the journal and to evaluate adherence to word limits and journal format. Papers also are assessed for originality, scientific quality, environmental health significance, clarity of presentation, and conciseness. Before papers are sent for peer review, they are screened for possible plagiarism (see Scientific Integrity below), and authors must submit a Competing Financial Interests Declaration form on behalf of all authors (see Competing Financial Interests below). Papers selected for review are assigned to an Associate Editor, who identifies reviewers and makes recommendations to the Editor-in-Chief. Members of the Editorial Review Board serve as a pool of potential reviewers of papers. Both the Board of Associate Editors and the Editorial Review Board are composed of leading scientists from all segments of the environmental health sciences. The overall acceptance rate of papers submitted to the journal is approximately 15%.

4.1.1. Types of Manuscripts

Manuscripts in the categories below are considered for publication. All manuscripts are peer reviewed except Correspondence. See Article Length below for details concerning word limits.

Correspondence (≤ 750 words) should address specific scientific issues or questions raised by Research or News Articles published in the journal within the previous 6 months. Authors of papers cited in Correspondence will be given the opportunity to respond. Letters addressing issues raised in previously published letters are discouraged. Correspondence may include a brief table or small figure if it is critical to the discussion. New data must not be included. Authors may include data from or redrawing of previously published materials as long as the work is cited and written permission from the original authors and/or publishers has been granted for republication in both printed and electronic form. Each figure is considered equivalent to 250 words toward the total word count. Correspondence that cites abstracts or unpublished observations is not acceptable

and will not be published. Letters that are highly polemic or personal in nature will not be published. Correspondence is not peer reviewed and is published at the discretion of the *EHP* editors. Conclusions and opinions expressed by the authors do not necessarily reflect the policies of *EHP*.

Commentaries (≤ 5,000 words) present information and personal insight on a particular topic. Commentaries should not be extended critiques of single articles appearing in *EHP* or elsewhere. Factual data should be included to substantiate arguments. *EHP* reserves the right to reject Commentaries without review if they are perceived as being too polemic or personal in nature. *EHP* also reserves the right to propose that Commentaries be reviewed as one side of a point/counterpoint debate. Assuming the original author agrees, *EHP* will ask another author to address the opposite side of an argument. If both papers are accepted, *EHP* will publish them together. Manuscripts on ethical, legal, social, or policy issues may also be accepted in this category.

Research Articles (≤ 7,000 words) report original scientific research and discovery. Research Articles may come from any field of scientific research relevant to the study of human health and the environment.

Substantive Reviews (≤ 10,000 words) provide an overview, integration of information, and critical analysis of a particular field of research or theme related to environmental health sciences. Previous research should be comprehensively reviewed regardless of whether the findings are consistent with expectations or the review authors' hypotheses. It is appropriate for authors to discuss the strengths and weaknesses of individual studies, focus on high-quality studies that add to the weight of the evidence on the topic under review, identify information gaps, and make recommendations for future research. Lengthy historical perspectives generally are not appropriate.

Quantitative Reviews and Meta-Analyses (≤ 10,000 words) present, contrast, and (when appropriate) combine data across studies to address a specific study question related to environmental health. Inclusion criteria and strategies used to search the literature should be explicitly described, along with analytic methods used to evaluate or combine data. The potential for publication bias and heterogeneity among studies should be investigated, and graphical displays of data contributed by individual studies are encouraged. The strengths and weaknesses of individual studies and potential causes of discordant findings among studies also should be discussed. As with Substantive Reviews, authors should integrate and critically analyze information from previous research, identify information gaps, and make recommendations for future research.

Reviews Based on Meetings or Conferences (≤ 10,000 words) should review the state of the science for a particular area, identify research gaps and needs, and explain how the outcome of

the meeting or conference addresses those gaps and needs. These reviews should focus on the science or theme but not on the conference or meeting itself. *De novo* data, participant lists, dialogue of workgroups or committees, and discussion of the internal organization of the meeting are not allowed. These papers should be submitted to *EHP* no more than 1 year after the meeting or conference takes place. Prospective authors should consult with the Editor-in-Chief before submitting a review based on a meeting or conference.

Brief Communications (≤ 4,000 words) are short scholarly reports that provide timely information of interest to the broad environmental health community. They may be used to highlight the importance of new environmental health programs or agencies or the advantages of new research approaches in the context of knowledge gaps; or to raise awareness of and make recommendations for addressing contemporary or emerging environmental health problems. A Brief Communication may take the form of a statement from an organization or group concerning the need for action on an environmental health issue (typically with recommendations). Authors should contact the Editor-in-Chief in advance for permission to submit. Brief Communications are reviewed internally for relevance, importance, and clarity, and are published without Advance Publication in the Perspectives section of *EHP*. They are assigned a DOI number and indexed in PubMed/MEDLINE. Formatting requirements, including references and any tables or figures, are consistent with those for *EHP* Research Articles, with the exception of the abstract, which must be unstructured (without subheadings) and ≤ 200 words. In addition, Supplemental Material is not allowed.

4.1.2.

4.1.3. Originality of Submission

Contributions submitted to *EHP* must be original works of the author(s) and must not have been previously published in print or online or simultaneously submitted to another publication. Previously published material (e.g., figures, tables) may be included in Commentaries and Reviews, assuming the original authors have given permission to reproduce the material and all copyright issues have been resolved. For original Research Articles, previously published schemata or illustrative figures are acceptable with the proper attribution and permission. Text or narrative from guidance documents, technical reports, and position papers by various government and nongovernmental organizations may be considered if they include new information.*EHP* will consider papers from dissertations that have been published in their entirety by a university in partial fulfillment of a degree. Manuscripts presented at a scientific meeting but not published in full or under review for publication elsewhere also will be considered. As indicated in *Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication* [International Committee of Medical Journal Editors (http://www.icmje.org/urm_full.pdf)], it is the responsibility of the author to make a full statement to

the editor concerning materials in a manuscript that might be considered redundant or duplicative. For additional clarification, please contact the Editor-in-Chief.

4.1.4.

4.1.5. Scientific Integrity

EHP requires assurances that animals used in a study have been treated humanely and with regard for the alleviation of suffering. Research involving humans must have been conducted according to the Common Rule (http://ori.dhhs.gov/education/products/ucla/chapter2/page04b.htm). Research involving humans also must be approved by an appropriate institutional review board and comply with all relevant national, state, and local regulations. For research conducted outside the United States and thus exempt from U.S. federal regulations, authors must perform the research in accordance with principles of the Declaration of Helsinki (http://www.wma.net/en/30publications/10policies/b3/). Approval and compliance with research requirements regarding human subjects must be noted, and information regarding informed consent procedures must be described in the "Methods" section of manuscripts concerning human subjects research.

EHP is sometimes confronted with issues regarding potential research misconduct, such as plagiarism or data fabrication. Authors should be aware that all papers submitted to *EHP* are screened routinely for plagiarism, defined as "the appropriation of another person's ideas, processes, results, or words without giving appropriate credit" (American Medical Association. 2007. *AMA Manual of Style: A Guide for Authors and Editors*, 10th edition. New York:Oxford University Press). Instances of documented plagiarism and allegations of data fabrication will be brought to the attention of the authors' host institutions. Documented cases of plagiarism or data fabrication could lead to a 3-year ban on future publication in *EHP* by the authors, a published Expression of Concern, and/or retraction of the paper.

4.1.6. Dual-Use Research

EHP anticipates receiving submissions on research that, based on current understanding, can be reasonably anticipated to provide knowledge, products, or technologies that could be directly misapplied by others to pose a threat to public health and safety, agriculture, plants, animals, or the environment (also known as dual-use research). Papers flagged for dual-use issues by *EHP* editors will undergo an additional level of review concerning the implications to society of publishing such a paper, and *EHP* reserves the right to seek expert advice in such cases. Authors should be aware that *EHP* could determine that the risks to public health and safety of publishing the paper outweigh the benefits of publishing, even if the paper has otherwise been deemed acceptable for publication.

4.1.7.

4.1.8. Suggested Guidelines

EHP endorses the ARRIVE guidelines for reporting results from animal studies (http://www.nc3rs.org.uk/ARRIVE). We encourage authors to review these guidelines when designing their studies and to use them in writing papers for submission to *EHP*, and we encourage our Associate Editors and peer reviewers to keep in mind the principles articulated in the ARRIVE guidelines when evaluating papers involving animal research. *EHP* encourages authors of Review articles to follow recommendations for transparent reporting of systematic reviews as described in the PRISMA Statement (http://www.prisma-statement.org). Authors performing microarray experiments should follow the Minimum Information About a Microarray Experiment (MIAME) guidelines developed by the Microarray Gene Expression Data (MGED) Society (http://www.mged.org/miame).

4.1.9.

4.1.10. Competing Financial Interests

EHP has a policy of full disclosure. Authors must declare all actual or potential competing financial interests involving people or organizations that might reasonably be perceived as relevant. Disclosure of competing interests does not imply that the information in the article is questionable or that conclusions are biased. Decisions to publish or reject an article will not be based solely on a declaration of a competing interest.

For each manuscript, authors must submit a Competing Financial Interests Declaration (CFID) form (available at http://ehp.niehs.nih.gov/wp-content/uploads/2012/09/EHP-CFI-form-blank.pdf). Papers will not be processed for peer review unless a CFID form has been submitted. Authors of Correspondence and Editorials also are required to submit a CFID form.
Authors must disclose all actual or potential competing financial interests occurring within the last 3 years, including but not limited to

- Grant support
- Employment (past, present, or firm offer of future)
- Patents (pending or applied)
- Payment for expert witness or testimony
- Personal financial interests by the authors, immediate family members, or institutional affiliations that may gain or lose financially through publication of the article
- Forms of compensation, including travel funding, consultancies, board positions, patent and royalty arrangements, stock shares, or bonds. Diversified mutual funds or investment trusts do not constitute a competing financial interest. Authors should carefully examine the wording of

documents such as grants and contracts to determine whether there might be an actual or potential competing interest.

Employment of any author by a for-profit or nonprofit foundation or advocacy group or work as a consultant also must be indicated on the CFID form.

As a condition of review and publication, authors must further certify that their freedom to design, conduct, interpret, and publish research is not compromised by any controlling sponsor.

A statement of disclosure consistent with the information contained in the CFID form must be included in the Acknowledgments section of the manuscript submitted to the journal. If there are no actual or potential competing financial interests, this must be indicated: for example, "The authors declare they have no actual or potential competing financial interests."

Editors and reviewers also must disclose to the Editor-in-Chief any actual or potential competing interests, both financial and nonfinancial, that have occurred within the last 3 years and could reasonably be perceived as relevant. Competing nonfinancial interests include former or current mentor–student relationships, faculty appointments in the same department or organization, familial relationships, service on advisory boards that oversee the research under review, collaborations, or membership in organizations that hold ideological views that are contradictory to the theme or topic under review.

EHP relies on the integrity of all authors to provide accurate disclosure statements. However, authors can expect scrutiny of their statements by the editors, reviewers, and readership. Alleged inaccuracies of declared competing interests should be addressed to the Editor-in-Chief. *EHP* will impose a 3-year ban on publication in *EHP* by any authors found to have willfully failed to disclose a competing financial interest. A paper may also be retracted or an Expression of Concern published and appended to the article.

5. MANUSCRIPT PREPARATION

5.1.1.

5.1.2. Article Length

All words in the main text, title pages, abstract, tables, and references count toward *EHP* word limits. In addition, each figure is counted as 250 additional words. Manuscripts that do not conform to the word limits may be returned to the author(s) for revision before the review process is initiated. Depending on the topic and potential impact of a paper, the Editor-in-Chief reserves the right to waive word limits. Authors may place some types of information, such as lengthy descriptions of

previously published methods, into Supplemental Material; however, these methods must be described briefly in the text of the paper. Information included in Supplemental Material does not count toward the word limit. The judicious use of references also may help meet the following word limits:

- Correspondence: ≤ 750 words
- Commentaries: ≤ 5,000 words
- Research Articles: ≤ 7,000 words
- Substantive Reviews: ≤ 10,000 words
- Quantitative Reviews and Meta-Analyses: ≤ 10,000 words
- Reviews Based on Meetings or Conferences: ≤ 10,000 words.

5.1.3.

5.1.4. Parts of a Manuscript

Title Pages

The title pages should include the following items in the order shown, beginning on the first page of the manuscript:

- Manuscript title, not to exceed 20 words [Titles should describe the research or topic of the paper but not summarize results or conclusions; titles generally should not contain abbreviations or numerical values, with the exception of abbreviated study names (e.g., NHANES)]
- Names of the authors spelled out in full
- Affiliations of all authors (department, institution, city, state/province, and country)
- Name of and contact information for corresponding author to whom page proofs should be sent, including complete address for express mail service, telephone number, and e-mail address
- A short running title, not to exceed 50 characters and spaces
- Acknowledgments, including grant information
- A competing financial interests declaration.

Abstract

All papers must include a structured abstract of ≤ 250 words, which should not contain references. No information should be reported in the abstract that does not appear in the text of the manuscript. In general we recommend that authors indicate study names or sources of data that are integral to the study in the title or abstract. Conclusions should mention the relevance of the work to environmental health science. Headings to be used in the structured abstracts vary by article type as described below:

- Commentaries: Background, Objectives, Discussion, Conclusions
- Research Articles, Quantitative Reviews, and Meta-Analyses: Background, Objectives, Methods, Results, Conclusions
- Substantive Reviews and Reviews Based on Meetings or Conferences: Background, Objectives, Methods, Discussion, Conclusions.

Main Text

The organization of the text varies by article type and roughly reflects the structure of the abstract:

- Commentaries: Introduction (comprising the Background and Objectives stated in the abstract), Discussion, Conclusions
- Research Articles: Introduction (comprising the Background and Objectives stated in the abstract), Methods, Results, Discussion, Conclusions
- Reviews: Introduction (comprising the Background and Objectives stated in the abstract), Methods (including data sources), Results (as appropriate), Discussion, Conclusions.

Concise subheadings (≤ 8 words each) may be used to designate major topics within each of these sections.

References, Tables, Figures, and Supplemental Material

The following items should be provided after the main text of the paper in this order: References, Tables, Figure Legends. The References, Tables, and Figure Legends must each begin on a new page of the manuscript. Figures and Supplemental Material should be provided as separate files. Additional information concerning each of these sections is provided in "*EHP* Style" below.

5.1.5.

5.1.6. Conformance to ***EHP*** Style Guidelines

Manuscripts submitted to *EHP* must conform to all *EHP* style requirements as described in "*EHP* Style" below. Authors should take special note of requirements for citations and references, figures, and tables. Manuscripts that do not conform to style requirements may be returned to the authors for modification before the initiation of the peer-review process. This step will cause a significant delay in the review and possible acceptance of the manuscript. All manuscripts must be submitted to *EHP* in English.

5.1.7.

5.1.8. Manuscript Formatting

Manuscript pages must be numbered consecutively, beginning with the title page, and lines should be numbered in the original submission and all subsequent revisions. The manuscript must be prepared using Times New Roman font at 12-point size. The manuscript must be double-spaced, with all margins set at 1 inch.

For additional information, see the *AMA Manual of Style: A Guide for Authors and Editors*, 10th edition (American Medical Association 2007). A basic source for spelling is *Merriam-Webster's Collegiate Dictionary*, 11th edition.

Resources for assistance with research, presentation, and language are available from the following organizations:

- International Committee of Medical Journal Editors [*Uniform Requirements for Manuscripts Submitted to Biomedical Journals: Writing and Editing for Biomedical Publication*(http://www.icmje.org/)]
- AuthorAID (http://www.authoraid.info/).

6. *EHP* STYLE

6.1.1.

6.1.2. Plain Language

EHP covers all disciplines engaged in the broad field of environmental health sciences. Therefore, authors should write in a clear and simple manner, in the active voice, and avoid unnecessary jargon, so the article is understandable to readers in other disciplines and to those whose first language is not English. In deference to the breadth of the journal's readership, please define terms that may not be universally recognized among all environmental health scientists.

Clearly define all outcomes, exposures, predictors, confounders, and covariates, and describe the methods or assays used to characterize study data. Results should be presented in a clear and unambiguous manner. Comparison groups or reference conditions should be clearly indicated when reporting measures of association or effect and when reporting *p*-values for statistical tests comparing outcomes or effects between groups.

We recommend against the use of "-fold" terminology because it can be difficult to determine whether it is being used to describe relative versus absolute differences or changes between groups or conditions.

Whenever possible, provide an estimate of variability or precision when reporting measures of association or central tendency (e.g., confidence intervals, standard deviations, interquartile ranges), regardless of whether *p*-values are also reported for these estimates.

6.1.3.

6.1.4. Abbreviations

All abbreviations, including abbreviations for elements (e.g., Fe, Cu) and chemical compounds [e.g., polychlorinated biphenyls (PCBs), carbon dioxide (CO_2)], should be defined in the text on first use with abbreviations used thereafter.

Units of measure should be abbreviated only when a specific amount is given (e.g., “concentration of 10 ng/mL” versus “units of nanograms per milliliter”).

6.1.5. In-Text Citations and Reference Lists

References and citations must be formatted according to *EHP* style as described below. This will reduce copyediting time and the number of author queries included in page proofs. Authors should double-check all references for accuracy and completeness of information, spelling, diacritical marks, symbols, subscripts/superscripts, and italics. Authors are fully responsible for the accuracy of their references.

In-Text Citations

All in-text citations must be in name/date form. Place the citation immediately after the textual information cited, placing name and date within parentheses without a comma. EndNote is a useful source for *EHP* reference style; the current *EHP* reference style for EndNote can be downloaded from http://www.endnote.com/support/enstyles.asp.

- Single author: (Wing 2002)
- Two authors: (Wing and Wolf 2000)
- Three or more authors: Use first author’s last name plus “et al.” (Wing et al. 2008)
- Multiple sources cited at one time: List publications alphabetically by author in the citation. Separate publications by the same author(s) with commas and those by different authors with semicolons: (Aldridge et al. 2005; Jameson et al. 2006; Levin et al. 2007; Slotkin 2004a, 2004b; Slotkin et al. 2008)
- Multiple sources cited at one time with different first authors but same last name and date: Use first author’s last name plus initial(s) (Smith A 2000; Smith J 2000).

Provide references for any quotations used in the text. For example:

According to Rubin et al. (2001), “it is only with a multidisciplinary and collaborative approach that the environmental and public health significance of *Pfiesteria* will be fully understood.”

Any items that must be cited but are not accessible to the public must appear in the text in parentheses but should not be listed in the references: (Ramsdell JS, Moeller PDR, personal communication); (Reeves MK, unpublished data).

Reference List

Authors are fully responsible for the accuracy of their references. The list of references should begin on a new page after the Conclusions of the manuscript. All references must include

- Author/editor last name plus initials (for six or fewer authors; if there are more than six authors, use “et al.” after the sixth) or authoring agency
- Year of publication
- Full title of article or chapter (lower case)
- Title of journal [abbreviated according to BIOSIS, *Index Medicus*, or PubMed (http://www.ncbi.nlm.nih.gov/nlmcatalog/journals)] or book/proceedings in title case
- For books and meeting reports, city/state/country of publication and name of publisher
- Volume and inclusive page numbers
- DOI number, if available; this information is required for articles published online only
- For websites and documents available online, URL (web address) and date accessed.
  If you are uncertain what to include, please include all information.

List references alphabetically by the last name of the first author. If the first author has more than one publication, list references in alphabetical order (letter by letter) of subsequent authors. If the first author shares the last name with another first author (Smith JM vs. Smith RB), alphabetize by initials. If you list more than one publication by the same author/group of authors, arrange publications by date, early to late. If you list more than one publication published in the same year by the same author/group of authors, use a, b, c, and so on to distinguish the publications.

Sample Alphabetical List

Slotkin TA. 2004a. Cholinergic systems in brain development and disruption by neurotoxicants: nicotine, environmental tobacco smoke, organophosphates. Toxicol Appl Pharmacol 198:132–151.

Slotkin TA. 2004b. Guidelines for developmental neurotoxicity and their impact on organophosphate pesticides: a personal view from an academic perspective. Neurotoxicology 25:631–640.

Slotkin TA. 2005. Developmental neurotoxicity of organophosphates: a case study of chlorpyrifos. In: Toxicity of Organophosphate and Carbamate Pesticides (Gupta RC, ed). San Diego:Elsevier Academic Press, 293–314.

Slotkin TA, MacKillop EA, Ryde IT, Tate CA, Seidler FJ. 2007. Screening for developmental neurotoxicity using PC12 cells: comparisons of organophosphates with a carbamate, an organochlorine and divalent nickel. Environ Health Perspect 115:93–101.

Slotkin TA, Persons D, Slepetis RJ, Taylor D, Bartolome J. 1984. Control of nucleic acid and protein synthesis in developing brain, kidney, and heart of the neonatal rat: effects of a difluoro-methylornithine, a specific, irreversible inhibitor of ornithine decarboxylase. Teratology 30:211–224.

Slotkin TA, Seidler FJ. 2007. Comparative developmental neurotoxicity of organophosphates in vivo: transcriptional responses of pathways for brain cell development, cell signaling, cytotoxicity and neurotransmitter systems. Brain Res Bull 72:232–274.

6.1.6.

6.1.7. Types of references

Journal article—conventional reference

Lewin SW, Arthur JR, Riemersma RA, Nicol F, Walker SW, Millar EM, et al. 2002. Selenium supplementation acting through the induction of thioredoxin reductase and glutathione peroxidase protects the human endothelial cell. Biochim Biophys Acta 1593:85–92.

Journal article—advance publication

Fanshawe TR, Diggle PJ, Rushton S, Sanderson R, Lurz PWW, Glinianaia SV, et al. 2007. Modelling spatio-temporal variation in exposure to particulate matter: a two-stage approach. Environmetrics; doi:10.1002/env.889 [Online 17 December 2007].

Journal article—published online only

Cazelles B, Chavez M, McMichael AJ, Hales S. 2005. Nonstationary influence of El Niño on the synchronous dengue epidemics in Thailand. PLoS Med 2:e106; doi:10.1371/journal.pmed.0020106.

Journal article, “in press”

Theppeang K, Glass TA, Bandeen-Roche K, Todd AC, Rohde CA, Schwartz BS. In press. Sex and race/ethnicity differences in lead dose biomarkers: predictors of lead in blood, tibia, and patella in older, community-dwelling adults in an urban setting. Am J Public Health.

Chapter in edited book

Clark K, Cousins I, MacKay D, Yamada K. 2003. Observed concentrations in the environment. In: The Handbook of Environmental Chemistry, Vol 3, Part Q: Phthalate Esters (Staples CA, ed). New York:Springer, 125–177.

Agency as author

National Research Council. 2011. Guide for the Care and Use of Laboratory Animals. 8th ed. Washington, DC:National Academies Press.

Proceedings

Zaslavsky I, Pezzoli K, Valentine D, Lin A, Sarabia H, Ellisman MH, et al. 2006. Integrating GIS and portal technologies for assessing environmental health impacts of Hurricane Katrina. In: Proceedings from the Second International Conference on Environmental Science and Technology, 19–22 August 2006, Houston, TX, Vol 2 (Starrett SK, Hong J, Lyon WG, eds). Houston, TX:American Science Press, 385–390.

Web document

NTP (National Toxicology Program). 2008. NTP-CERHR Monograph on the Potential Human Reproductive and Developmental Effects of Bisphenol A. NIH Publication no. 08-5994. Available: http://cerhr.niehs.nih.gov/evaluations/chemicals/bisphenol/bisphenol.pdf [accessed 24 June 2010].

Website

CDC (Centers for Disease Control and Prevention). 2013. National Health and Nutrition Examination Survey Homepage. Available: http://www.cdc.gov/nchs/nhanes.htm [accessed 5 November 2013].

(Additional reference samples are available below.)

6.1.8.

6.1.9. Footnotes

Do not use footnotes. Place all textual information within the manuscript and all references in the proper form both in text and in the reference list.

6.1.10.

6.1.11. Preparing Tables and Figures

Tables

Each table must begin on a new page after the References. Tables must be numbered with Arabic numerals, followed by a brief title (not to exceed 25 words). Tables should contain no more than two layers of column headings. A column heading must be provided for each column. Additional column heads should not be placed in the middle of a table. Tables must be created using the Table feature in Microsoft Word. List abbreviations and definitions under each table. Type footnotes directly after the abbreviations, beginning on the next line. General footnotes to tables

must be indicated by lowercase superscript letters beginning with “a” for each table. Footnotes indicating statistical significance must be identified in the following order: asterisks (*, **), number signs (#, ##), and daggers (†, ††). The comparison to which the *p*-value applies must be clearly indicated (e.g., “compared with untreated controls”). For presentation of data in tables, please use the “±” symbol for arithmetic mean and standard deviation or standard error (e.g., “mean ± SE”) and parentheses for the standard error when presented with the geometric mean [e.g., “GM (SE)”]. Please present number and percent as “*n* (%)” (i.e., in one column separated with one space). Confidence intervals should be presented in parentheses in the same column as the point estimate, with the upper and lower bounds separated by a comma [e.g., (0.1, 2.3)].

Figure Legends

Figure legends should be provided on a new page after tables. Each figure legend should include a title for the entire figure and descriptors for each panel [e.g., “Figure 1. Incidence of hepatocellular adenomas (*A*) and carcinomas (*B*) in mice exposed to DEHP”]. Define error bars and any abbreviations not defined in the text. Footnotes indicating statistical significance must be identified in the following order: asterisks (*, **), number signs (#, ##), and daggers (†, ††). The comparison to which the *p*-value applies must be clearly indicated (e.g., “compared with controls from the corresponding age group”). Type footnotes directly after the abbreviations beginning on the next line.

Figures

Each figure must be provided as a separate file in one of the following formats: EPS, PDF, TIFF, or JPG. Do not embed figures in the main text (Microsoft Word) file. Each figure must be labeled with the figure number. For TIFF or JPG format, the resolution should be 300 dpi for color images, 600 dpi for grayscale images, and 1,200 dpi for line art (black-and-white art). JPG files should be saved on the “highest quality” setting. Color images should be RGB and saved at a minimum of 8 bits per channel. Because figures may be reduced or enlarged to fit our layouts, sufficient resolution is essential. Vector images should be saved as editable EPS files. Any images embedded in the EPS should also be included in a separate file. Do not convert text to path outlines before submission.

Graphics must fit standard letter-size paper (8.5 × 11 inches, portrait orientation). Multiple panels within a figure also must fit on a single page. All letters, numbers, and lines must be clearly legible and easy to differentiate. Provide a key defining representational elements (e.g., dotted/dashed lines, symbols, box plot elements) for each figure. All axes must be clearly labeled, giving both the measure and the unit of measurement where applicable. Consistency among terms and styles

(including symbols and colors) used in figures is desirable. For example, if a black circle represents the control in Figure 1, a black circle (or a black bar) should be used for controls in all other figures. Photomicrographs should include a scale bar in each image, and the length should be specified in the typed figure legend (e.g., “bar = 10 μm”). *EHP* encourages authors to use color to enhance figures. However, to ensure accessibility, all figures must be interpretable when printed in black and white.

EHP editors reserve the right to request that complex figures (e.g., figures with multiple panels showing information in a variety of formats, or that include panels related to different experiments) be divided into separate figures for publication. Questions concerning figures should be directed to EHPmanuscripts@niehs.nih.gov.

Image Integrity

Adjusting an image for brightness and contrast is acceptable if it is applied to the entire image. Background data of gels and blots must not be removed. The final image must accurately represent the original data.

6.1.12.

6.1.13. Supplemental Material

EHP welcomes reasonable amounts of material suitable for inclusion as online documentation for submitted manuscripts. Examples are bioinformatic data, formulae, statistical derivations, full gene data and analysis, additional high-resolution microscopic data, kinetic analyses, and other supporting tables, figures, or videos. The submitted manuscripts must be able to stand alone in the absence of Supplemental Material. All information included as Supplemental Material should be directly relevant to the article; however, information should be included only in the paper or the Supplemental Material—not in both. The principal methodological approach must be clearly described in the main body of the paper and not relegated to Supplemental Material. Supplemental Material will be peer reviewed along with the manuscript and thus must meet the same rigorous standards.

Supplemental Material should not exceed 2,000 words, including text, tables, references, and figure legends plus an additional 250 words per figure. If the Supplemental Material exceeds this limit, the author must justify the overage in the Cover Letter required at submission. Authors may provide a separate (permanent) web repository for information that is not included in the Supplemental Material file if they believe it would be of interest to readers. This material should be

clearly identified as not peer reviewed. This information should be cited in the text and included in the reference list (formatted as a website).

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- Begin the Supplemental Material file with a title page that indicates "Supplemental Material" followed by the title of the paper and the author list.
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- Place figure legends below corresponding figures.
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- Tables or figures included in the Supplemental Material should be labeled as Table S1; Figure S1; and so on.
- When referring to Supplemental Material in the main manuscript, indicate the table, figure, or section as follows: See Supplemental Material; see Table S1; see Fig. S1; see Supplemental Material, p. 6; see Supplemental Material, Part 2.
- A separate reference list must be included in the Supplemental Material file for any sources cited in the Supplemental Material, even if they are cited in the main paper.

7. MANUSCRIPT SUBMISSION

7.1.1.

7.1.2. Editorial Manager

Manuscripts submitted to *EHP* will be processed using Editorial Manager, an online manuscript submission and tracking program (http://www.editorialmanager.com/ehp).

7.1.3.

7.1.4. Initial Submission of a Manuscript

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7.1.5.

7.1.6. Required Cover Letter

A cover letter must accompany the manuscript and include the following points:

- Assurances that the manuscript *a*) is an original work, *b*) has not been previously published whole or in part, and *c*) is not under consideration for publication elsewhere
- A statement that animals used in research have been treated humanely according to institutional guidelines, with due consideration to the alleviation of distress and discomfort. The source of those guidelines must be provided
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- The names and e-mail addresses of up to six possible preferred reviewers, as well as up to six nonpreferred reviewers for the manuscript
- If applicable, a statement concerning previous publication of a manuscript or materials that might be considered redundant or duplicative.

7.1.7. Peer Review

Manuscripts are assessed for originality, scientific quality, environmental health significance, clarity of presentation, and conciseness. Scientific quality and environmental significance have a higher weight than the other criteria.

All new submissions undergo an initial review by a group of consulting editors. Authors will be notified within 1–3 weeks if their paper is not selected for peer review based on the evaluation. Papers selected for peer review (~ 20% of new submissions) are assigned to an Associate Editor, who identifies at least two peer reviewers and makes recommendations to the Editor-in-Chief based on their reviews. Reviewer names are not provided to authors, but author names are available to reviewers. Reviewers are asked to complete their review within 2 weeks.

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7.1.8.

7.1.9. Resubmission of a Revised Manuscript

If *EHP* requests revisions or accepts the manuscript, authors will need to submit all of the following through Editorial Manager (http://www.editorialmanager.com/ehp):

- All text, tables, and figure legends must be in one Microsoft Word document. Please ensure that any symbols and equations appear correctly on printed copies and that all figures and tables are cited in the body text in numerical order.
- Each revised figure must be submitted as a separate file in one of the following formats: EPS, PDF, TIFF, or JPG. Each figure must be labeled with the correct figure number for the revised manuscript. Additional information on formatting and content requirements is provided in "Preparing Tables and Figures" above.
- Authors must submit a cover letter with point-by-point responses to the reviewers' comments, a copy of the revised manuscript with changes tracked in Microsoft Word, and a clean version of the revised manuscript with all changes accepted. To expedite the processing of revised manuscripts, it is important to be as specific as possible in responding to reviewers' comments. Authors should copy the editors' and/or reviewers' comments into the response letter and respond to each comment individually, including the specific changes made in response to each comment (if any) and where the changes are located in the revised draft. As in the original submission, the revised manuscripts must have numbered lines to facilitate locating specific text or changes.
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7.1.10.

7.1.11. Final Internal Review

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8. PUBLICATION SEQUENCE

8.1.1.

8.1.2. Advance Publication

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8.1.3.

8.1.4. Copyediting/Page Proofs

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8.1.7.

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