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Longitudinal Assessment of Chlorpyrifos Exposure and Self-Reported Neurological Symptoms in Adolescent Pesticide Applicators

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ABSTRACT

Objectives: Occupational exposure of organophosphorus pesticides (OPs) such as chlorpyrifos (CPF) in adolescents is of particular concern because the potential vulnerability of the developing neurological system. The objectives of the study were to examine how neurological symptoms reported over the CPF application season vary across time, whether these effects are reversible post application and if there are any associations between CPF biomarkers and neurological symptoms in an adolescent study population.

Methods: Egyptian adolescent CPF applicators (n=57) and non-applicators (n=38) were recruited for a longitudinal study. Self-reported data for 25 neurological symptoms were collected at 32 time points over the 7-month period before, during and after CPF-application. Urine and blood samples were collected for CPF-specific biomarkers urinetrichloro-2-pyridinol (TCPy), and blood cholinesterase.

Results: We observed increased reporting of neurological symptoms among both applicators and non-applicators after several weeks of repeated CPF application. Applicators demonstrated a greater percentage of neurological symptoms relative to baseline than the non-applicators after accounting for potential covariates. Similar models revealed that cumulative TCPy was positively and significantly associated with the average percentage of symptoms, but only among the applicators. Associations of the change butyrylcholinesterase (BChE) from pre to post application season with several subclasses of symptoms were also found significant or marginally significant.

Conclusions: These observations reinforce the growing concern regarding the neurotoxic health effects of CPF in adolescents and the importance of exposure prevention during the application season.

STRENGTHS AND LIMITATIONS OF THE STUDY:

- This is the first longitudinal study showing the association between specific organophosphorus pesticide exposure and reporting of neurological symptoms in adolescent applicators.
- Symptoms in applicators are compared with symptoms in non-applicator thus showing the effect of environmental CPF exposure in general population.
- The study is also novel in its approach to include prospective measures of biomarkers of CPF exposure and effect and to examine their associations with neurological symptoms.
- The non-specific nature of many of the symptoms is a limitation of the current study.
- Small sample size is another limitation study that may have influenced the significance levels of exposure-outcome relationships.
- Results of the study may be generalizable only to agricultural communities with similar sociodemographic characteristics.

What this paper adds

- It is not fully understood how neurological symptoms vary across time in adolescents exposed to specific organophosphorus pesticide.
- Applicators are more likely to report increased symptoms compared to non-applicators.
- Repeated occupational exposure to CPF increases the reporting of acute neurological symptoms during the CPF application season and the symptoms may persist for months after the cessation of exposure in both applicators and non-applicators.
- Cumulative biomarker of CPF exposure also demonstrates association with neurological symptoms in applicators.
- Reduction of CPF exposure among the adolescent applicators should be a public health priority since neurological symptoms remained elevated even after the cessation of CPF application.

INTRODUCTION

High prevalence of agricultural use of organophosphorus pesticides (OPs) has been recognized as a major global public health challenge for agriculture-based communities due to their associations with neurological outcomes. Immediate or short-term neurological signs and symptoms ranging from less severe (headache, dizziness, nausea etc.) to more severe (muscle weakness, bronchospasm, change in heart rate etc.) were all reported after occupational OP exposure¹. These short-term symptoms were reported as early as 48 hours after acute exposure². Although high levels of occupational OP exposure can be associated with symptoms persisting for several years³, repeated moderate to low exposures can also produce chronic neurological symptoms and deficits in neurobehavioral performance⁴. Converging

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evidence regarding the associations between OP exposures and neurological symptoms is based on adult occupational studies conducted in a wide range of study settings. These include comparisons between exposed and non-exposed farmworkers in the US⁵, South Africa⁶, Nicaragua^{7,8}, Kenya⁹, Sri Lanka¹⁰ and Egypt¹¹. These studies have used self-reported questionnaire data containing non-specific neurological symptoms. Additional evidence for the effect of pesticides on somatic and mood symptoms are also found in the literature^{2,12}.

Although less commonly studied, OP exposures were also found to be associated with neurological symptoms in children and adolescents. In developing countries children and adolescents are engaged in OP application and this presents a major public health concern¹³. Even in the US, adolescents can be involved in mixing and applying pesticides^{14,15}. Because of their smaller body size, the biological doses of pesticides (for children and adolescents may be substantially higher than adults¹⁶, making them more vulnerable to neurological effects. Animal and human studies have also suggested that paraoxonase (PON 1)—an organophosphate detoxifying enzyme—is less active in younger populations making them more vulnerable to OP toxicity^{17,18}. An Egyptian cross-sectional study found adolescent pesticide applicators reporting more neurological symptoms and neuromuscular problems than controls^{19,20}. Association between environmental OP exposure and neurological symptoms was also demonstrated in children living in an Indian agricultural community²¹.

Biomarkers have been used to characterize OP exposure in epidemiological and occupational studies. Urinary trichloro-2-pyridinol (TCPy), a relatively specific CPF metabolite of exposure, which is eliminated in the urine with a half-life of 27 hr following exposure²². Due to the ease and non-invasiveness of collection of urine samples, TCPy is widely recognized as a useful biomarker of exposure, particularly in children and adolescents^{23,24}. The classic mode of OP toxicity is manifested by the inhibition of cholinesterase. Both blood acetylcholinesterase (AChE) and butyrylcholinesterase

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3 (BChE) are biomarkers of effect with BChE being more sensitive to inhibition by OP pesticides²⁵. A
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5 small number of adult studies found associations between inhibition of cholinergic activities with self-
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7 reported symptoms^{9 10}; however, this relationship has rarely been examined in adolescent studies.
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12 Understanding the relationship between OP exposure and the change in neurological symptoms
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14 across time (temporal change) is important because application-related exposure follows a seasonal
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16 pattern in most areas. Moreover, specific OP exposure is important to track the changes in symptom
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18 reporting over time. Two longitudinal studies with agricultural workers demonstrated that short-term
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20 neurological signs and symptoms were associated with initial acute episodes of exposure, which
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22 eventually advanced into long-term sequelae^{7 12}. However, these studies did not characterize exposure
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24 and did not identify any specific OP that was being applied.
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29 To investigate whether occupational exposure to CPF is associated with self-reported neurological
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31 symptoms, we compared adolescent applicators exposed to CPF with adolescent non-applicators working
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33 and residing in Egypt through a prospective study. Typically, CPF is the primary insecticide used by
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35 pesticide applicators in Egyptian cotton fields, including adolescent applicators, and offered us a unique
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37 exposure environment with well characterized occupational exposure. The possibility of potential
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39 confounding effects of other neurotoxic pesticides was minimal because of limited use of other pesticides
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41 in the study area. We attempted to answer the critical questions of how repeated exposures to OP
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43 determines reporting of neurological symptoms, how neurological symptoms vary across time during the
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45 exposure season, if these effects could reverse at the cessation of exposure and whether there are any
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47 associations between OP biomarkers and neurological symptoms in the adolescent study population. A
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49 questionnaire was administered pre-, mid- and post-CPF application season to examine changes in self-
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51 reported symptoms across time.
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METHODS

Study area and population

A prospective study was conducted in Menoufia Governorate, Egypt from April 2010 to January 2011. Two of the nine districts of Menoufia, Al-Shohada and Berket El-Sabea were chosen randomly to conduct the study (Supplementary Figure 1). In Egypt, adolescents are hired seasonally to apply pesticides to cotton fields and the schedule of pesticide applications to the cotton crop is regulated by the Ministry of Agriculture. In the year of 2011, approximately 2100 liters of OPs were applied on approximately 5700 acres of cotton fields (personal communication with the Ministry of Agriculture). Chlorpyrifos is the primary OP applied in the districts of Menoufia governorate from mid-June to early August. Although there are slight variations in the timing of CPF application between the two districts (Supplementary Figure 2) the application patterns are consistent across field stations.. The typical workday was from 8am-12pm and from 3pm-7pm, six days per week. Because there is no regulation in Egypt for mandatory use of personal protective equipment (PPE), dermal exposure and inhalation were both considered as the potential route of exposure in this population¹⁹. Recently,²⁶ reported that dermal exposure and subsequent absorption through the skin accounted for 94-96% of the total dose of chlorpyrifos in Egyptian pesticide applicators.

Recruitment and data collection

Fifty-eight male adolescents aged 12-21, hired seasonally by the Ministry of Agriculture to spray pesticides in the cotton fields were recruited from two field stations in the Menoufia governate (i.e. Al-Shohada and Berket El-Sabea, field station 1 and field station 2, respectively). Forty adolescent non-applicators were recruited through convenience sampling (word of mouth, direct communication with utilizing contacts through the staff from the local Ministry of Agriculture) from the same districts as the applicators for the cotton crop. These adolescents never worked in the field as pesticide applicators. We excluded one adolescent from the final analysis due to inconsistency in participating in study activities and two other subjects for questionable sample integrity, resulting in a final sample size of 95 (57

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3 applicators and 38 non-applicators). Written informed consent was obtained from all participants and their
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5 legal guardian (for those under 18). All the subjects were monetarily compensated for their time during
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7 the questionnaire survey, medical examination and biological samples (~\$5 per visit). The study was
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9 approved by the OHSU IRB in June 2009, and by the Medical Ethics committee of the Faculty of
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11 Medicine, Menoufia University in July 2009.
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14 Data collection occurred at the primary field station for each district. Pesticides applicators and
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16 supervisors meet in the field stations, which also provides storage area for the pesticides and the
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18 equipment used for application.
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21 22 23 **Outcome assessment**

24 We developed a multiple time-point, 25-item, short-term neurological symptom questionnaire on
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26 the basis of the widely used Q16 questionnaire²⁷ and a modified version of the Q16 used in a previous
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28 study with licensed pesticide applicators²⁸. The 25 symptoms were grouped into six domains: behavioral,
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30 autonomic, cognitive, sensory, motor and non-specific temporary disability (Table 1). There were five
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32 frequency choices (0-4) for each symptom ranging from “never” (coded as 0) to “everyday of the week”
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34 (coded as 4). Since more than 90% of the responses were between 0-2 (1=once a week and 2=once in
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36 every 2-3 days) we recoded each of the symptom response to “0” or “never” and “1” or “at least once a
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38 week or more.” Beginning on June 2 of 2010 through January 2011 participants reported symptoms
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40 occurring in the past week through this symptom questionnaire administered 32 separate times, at least
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42 once per week) and spanning all relevant application periods in the season (pre-application, during
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44 application, and post-application). The number of positive responses was totaled for each person to yield
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46 a score ranging from 0–25; division by 25 produced the proportion of symptoms endorsed and this
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48 proportion was averaged across the 32 collection points to produce a season-level mean proportion of
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50 self-reported symptoms. Participants also completed a questionnaire at baseline addressing their socio-
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52 demographic status, household and occupational use of pesticides such as number of days of pesticide
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application or mixing, medical history, safety practices and lifestyle activities including smoking status, hours of sleep at night, number of drinks containing caffeine.

Table 1. Domains of neurological symptoms

Domains	Symptoms
Behavioral Symptoms	1. Tense or anxious [#]
	2. Excessively angry or irritable ^{*#}
	3. Depressed or withdrawn ^{*#}
Autonomic Symptoms	4. Nausea [#]
	5. Heavy sweating ^{*#}
	6. Loss of appetite [#]
	7. Fast heart rate ^{*#}
	8. Excessive salivation
Cognitive Symptoms	9. Difficulty concentrating ^{*#}
	10. Being absentminded and memory problem ^{*#}
Sensory Symptoms	11. Difficulty seeing at night [#]
	12. Blurred or double vision [#]
	13. Numbness in hands and feet [#]
	14. Sense of smell or taste change [#]
	15. Ringing in ears
Motor Symptoms	16. Difficulty with balance [#]
	17. Weakness in arms and legs [#]
	18. Involuntary movement of arms and legs [#]
	19. Shaking in hands [*]
Temporary Disability (non-specific symptoms)	20. Difficulty speaking [#]
	21. Dizziness [#]
	22. Headache ^{*#}
	23. Momentary loss of consciousness [#]
	24. Fatigue ^{*#}
	25. Insomnia [#]

*Symptoms used in Q-16²⁷

#Symptoms used in Agricultural Health Study²⁸

Urine collection and analysis

Urine was collected in wide mouth plastic cups at eight time points between April 2010 and January 2011. We collected spot urine samples at the field station at the beginning of the work shift. The cups were subsequently transferred to the laboratory at Menoufia University in a cooler with wet ice. At the laboratory, 4 ml aliquots of urine were transferred into labeled 5 ml cryovials within hours of sampling and stored at -20°C . The banked urine samples were express mailed on dry ice to University of Buffalo laboratory for analysis of pesticide metabolites; duplicate samples were retained in the -20°C freezer at Menoufia. Urine samples in the field station at Berket El-Sabea district were collected one day after the collection date of the field station at Al-Shohada.

The method of urinary TCPy measurement (a primary metabolite of chlorpyrifos) has been described elsewhere²³. Briefly, negative-ion chemical ionization gas chromatography–mass spectrometry was used that utilized ^{13}C - ^{15}N -3,5,6-TCPy as an internal standard. Jaffe reaction was used for colorimetric analysis of creatinine²⁹. The within-run imprecision of this assay is very low ($< 2\%$ coefficient of variation and an intra-class correlation coefficient of 0.997). The quality control (QC) samples consisted of lab samples that were first analyzed for TCPy levels; these levels were non-detectable. Twenty aliquots were then spiked with 50ng of TCPy/mL of urine; these were then extracted and analyzed as per protocol. The recovery rates ranged from 92% - 98% with the average being 94.8%, $\text{SD} = 0.931$ and the $\text{CV}\% = 1.965$, minimum detection level was 0.0501 ng. QC replicates had 94.75% recovery. Finally, cumulative urinary TCPy for each participant was determined by calculating the area under the curve for the plotted values for eight time intervals.

Blood collection and ChE analysis

To establish the baseline ChE activity, pre-application blood draws occurred on April 11 and June 2, 2010, prior to the start of the official government-regulated CPF application season. As with urine collection, blood draws in the field station at Berket El-Sabea were performed one day later. Changes in

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3 both AChE and BChE levels from baseline to the end of CPF-application season (blood collected on
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6 September 4, 2010) were estimated. Blood samples were collected by venipuncture into 10mL lavender
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8 top (EDTA) vacutainer tubes and immediately placed on wet ice and transported to Menoufia University,
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10 where they were analyzed in duplicate for AChE and BChE activity using an EQM Test-Mate kit (EQM
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12 Research Inc., Cincinnati, OH, USA) as described previously²³.
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14 15 16 17 **Statistical analysis**

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19 We used SPSS version 18.0 and STATA (version 11; Stata Corporation, College Station, TX) for
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21 the statistical analysis. Sociodemographic variables were summarized and described using means and
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23 standard deviations for continuous responses and percentages for discrete outcomes; simple comparisons
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25 between applicators and non-applicators were completed using t-tests (continuous measures) or chi-
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27 square tests (discrete outcomes). Concentrations of TCPy, AChE and BChE exhibited pronounced right
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29 skewness and more than a 3-fold separation between the minimum and maximum observed values;
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31 consequently, these responses were log transformed prior to analysis to improve symmetry. Both AChE
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33 and BChE were expressed as a log-transformed ratio of post-application activity relative to pre-
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35 application activity prior to investigation of associations with average percentage of self-reported
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37 symptoms. All p-values are two-sided with significance judged relative to a 0.05 level.
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43 Spearman correlation coefficients were used to estimate associations between urine and blood
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45 biomarkers and symptom scores. Self-reported neurological symptom counts were collected at 32
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47 irregularly spaced dates over an eight-month period spanning from early June 2010 through early January
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49 2011. These sample periods were collapsed into 10 separate non-overlapping intervals lasting between
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51 one and four weeks in length (Supplementary Figure 2). Symptom data from the first three dates (i.e first
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53 fifteen days of the study from June 2 to June 16), when no CPF was applied, was collectively taken to
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55 represent the baseline time interval (or time interval 1) against which symptom reporting from the other
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4 nine remaining time intervals was evaluated. In five of these nine time intervals (between June 19 and
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6 July 21), application of CPF was reported in both field stations. Generalized estimating equations (GEE)
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8 ³⁰ were used to model the proportion of neurological symptoms reported in each time interval while
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10 controlling for number of days worked (within five days of the symptom reporting date), home use of
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12 pesticides, age, education and income levels. The one fitted model was used to estimate changes over
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14 time, relative to the first time interval (June 2–June 16), for applicators and non-applicators as well as
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16 examine whether changes relative to baseline differed between the two groups (via group-by-time
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18 interaction).
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21 22 23 RESULTS

24 Sociodemographic Characteristics

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Ninety-two of the participants (97%) were between 12 and 18 years old with the remaining three between 19 and 21. The two groups, non-applicators and applicators, did not differ significantly in terms of age, educational status, family income, number of people in house, years of pesticide use at home, and insecticides and rodenticides use at home (Table 2). Compared to non-applicators, a significantly higher number of applicators lived close to the field (within 25 meters), had carpet in their homes and applied herbicides at home. Applicators had significantly lower BMI than non-applicators. On average, applicators had been working in the field for a little over 3 years.

Table 2: Sociodemographic characteristics for participants at baseline

Variables	Non-applicators (n=38)	Applicators (n=57)
	Mean (SD)	Mean (SD)
Age	16.6 (2.4)	16.2 (1.6)
Education	9.8 (1.8)	9.9 (1.8)
Height (cm) (34 non-applicators vs 30 app)	166.3 (12.0)	163.4 (10.0)
Weight (kg) (34 non-applicators vs 30 app)*	62.0 (15.4)	54.2 (8.6)
BMI (kg/m ²) (34 non-applicators vs 30 app)*	22.1 (3.7)	20.2 (2.2)
Number of people in house	5.6 (1.1)	6.0 (1.8)
Home pesticide use (years)* (19 Non-applicators & 44 App)*	1.6 (1.9)	2.5 (1.9)
Occupational application of pesticides (yrs)	-	3.1 (1.5)
Days/week of pesticide application	-	4.8 (1.3)
Hours/day of pesticide application	-	5.2 (0.7)
	% (n)	% (n)
Family Monthly Income (Low)	78.9 (30)	71.9 (41)
Applied pesticides in home in last 5 yrs (yes)*	47.4 (18)	78.9 (45)
Computer use (once a week or more)*	65.8 (25)	45.6 (26)
Carpet in house (yes)*	27.0 (10)	54.4 (31)
Live close to agricultural field (yes)*	23.7 (9)	50.9 (29)
Types of pesticides applied at home (24 vs 49)		
Herbicides*	13.0 (3)	44.9 (21)
Insecticides	83.3 (20)	93.9 (46)
Rodenticides	16.7 (4)	14.3 (7)

*p<0.05 for group difference

Change in symptoms over time

We considered day 0-14 as baseline time interval (1st time interval) when no application of CPF was reported. Applicators began increased reporting of neurological symptoms at the beginning of the chlorpyrifos application season (at the 2nd time interval between days 17-21 of the study). The percentage of neurological symptoms increased during the application season and reached the peak at the 6th time interval representing days 45-48, the time when the chlorpyrifos application period ended. The highest peak of symptom reporting was observed at the 8th time interval representing days 63-77 (Table 3).

Similar to the applicators, the non-applicators also demonstrated the highest increase in the proportion of neurological symptoms during the 8th time interval although the magnitude of the change was smaller (14 percentage point increase of symptoms relative to baseline interval). The change of neurological symptoms relative to baseline declined over the next two time intervals (9th and 10th) in both groups. For applicators, the percentage of reported symptoms at each of the nine subsequent time intervals was always higher than the percentage observed at baseline; non-applicators by contrast had a pattern of percentage of reported symptoms that both increased and decreased relative to baseline time interval over the course of the study (Figure 1).

When applicators and non-applicators are compared with respect to change in percentage of symptoms (relative to baseline), it was always the case that the change (percentage point change relative to baseline) for applicators was greater than the corresponding change for non-applicators even after adjusting for the covariates (Table 3).

Table 3. Estimated change (95% CI) from baseline in the percentage points of neurological symptoms reported at each of nine successive collected time points, shown separately for non-applicators and applicators.

	Time Intervals	Non-Applicators		Applicators		Change in difference between applicators and non-applicators relative to difference in baseline	
		Adjusted Models*	p-value	Adjusted Models*	p-value	b (% of Symptoms) (95% CI)	p-value
1	Days from Baseline (Day 0-14) June 2-June 14, 2010	b (% of Symptoms) (95% CI)	p-value	b (% of Symptoms) (95% CI)	p-value	b (% of Symptoms) (95% CI)	p-value
2	17-21 Jun 19-Jun 23	-2.74 (-4.61,-0.86)	0.004	4.08 (0.18,7.97)	0.040	6.81 (2.47, 11.15)	0.002
3	24-28 Jun 26-Jun 30	-2.68 (-5.18,0.17)	0.004	12.57 (7.75,17.38)	<0.001	15.25 (9.80, 20.69)	<0.001
4	31-35 Jul 3-Jul 7	4.45 (-0.80,9.71)	0.10	14.06 (9.21,18.90)	<0.001	9.60 (2.44, 16.77)	0.009
5	38-42 Jul 10-Jul 14	-2.28 (-4.75,0.17)	0.07	22.83 (19.25,26.40)	<0.001	25.11 (20.79, 29.43)	<0.001
6	45-48 Jul 17-Jul 21	0.46 (-2.37,3.30)	0.75	28.80 (24.27,33.35)	<0.001	28.35 (22.98, 33.72)	<0.001
7	52-59 Jul 24-Jul 31	4.95 (1.63,8.28)	0.003	24.01 (19.92,28.09)	<0.001	19.05 (13.80, 24.30)	<0.001
8	63-77 Aug 4-Aug 18	14.49 (11.45,17.55)	<0.001	30.10 (26.53,33.66)	<0.001	15.60 (10.95, 20.26)	<0.001
9	80-94 Aug 21-Sep 4	12.08 (8.72,15.44)	<0.001	29.17 (25.22,33.13)	<0.001	17.09 (11.93, 22.25)	<0.001
10	105-217 Sep 22-Jan 5 [#]	9.22 (6.12,12.32)	<0.001	18.45 (14.30,22.59)	<0.001	9.22 (4.12,14.33)	<0.001

*Estimates have been adjusted for number of days worked applying pesticides, home use of pesticides, age, education and income level. CPF application time intervals are shaded in grey.

Associations of neurological symptoms with biomarkers

TCPy was detected in 100% of the samples. Summary statistics for TCPy, AChE and BChE of the study samples have been already reported by ³¹, Mean creatinine concentration of the urine samples was reported to be 1696 µg/ml with maximum of 4199 and a minimum of 164 µg/ml. In brief, the applicators had much higher mean and estimated median peak TCPy concentration than the non-applicators (mean: 719 vs 44.9 µg/g creatinine; estimated median 137 vs 19.7 µg/g creatinine). In our study sample, BChE was found to be more sensitive to CPF exposure than AChE, with median activity reduced by 37% from baseline in applicators and 13% in non-applicators during the CPF application period.

A scatter plot of cumulative TCPy (ug/g creatinine) against average percentage points of symptoms revealed distinct exposure-response gradients by pesticide application status (applicator vs non-applicator) (Supplementary Figure 3a). In addition, two other scatter plots of change in AChE activity and change in BChE activity from pre-application to post-application against percentage of symptoms also revealed effect measure modification by pesticide application status (Supplementary Figures 3b & 3c). Therefore, separate linear models for applicators and non-applicators were used to examine the associations of these three biomarkers with the outcome measures.

Log-transformed TCPy was positively associated with the average percentage of neurological symptoms in the regression models after adjusting for field stations, age, family income, home pesticide use and average number of hours worked in the field among applicators ($b=2.68$, $p=0.007$). However, non-applicators demonstrated positive but statistically non-significant associations between TCPy and symptoms. Among applicators, AChE and BChE activity was negatively and significantly associated with the average percentage of neurological symptoms in the unadjusted models. In the adjusted models these associations remained negative but became non-significant (Table 4).

Table 4. Summary of regression analysis for biomarkers of exposure & effect of chlorpyrifos predicting average percentage of neurological symptoms over the entire study stratified by applicator status

Explanatory variables	Unadjusted Models			Adjusted Models*		
	B (se)	95% CI	p-value	B (se)	95% CI	p-value
<i>For Non Applicators</i>						
Ln TCPy (mg/g Cr) (n=28)	0.29 (0.76)	-1.26,1.84	0.71	0.57 (0.79)	-1.06,2.20	0.47
Ln (Post AChE/Pre AChE) (n=21)	-1.25 (16.41)	-35.59,33.1	0.94	-6.57 (18.80)	-43.64,33.50	0.73
Ln (Post BChE/Pre BChE) (n=21)	2.23 (7.04)	-12.51,16.98	0.76	2.50 (7.63)	-13.77,18.77	0.75
<i>For Applicators</i>						
Ln TCPy (mg/g Cr) (n=42)	4.56 (0.63)	3.29,5.84	<0.001	2.68 (0.93)	0.78,4.57	0.007
Ln (Post AChE/Pre AChE) (n=28)	-24.21 (12.79)	-50.50,2.09	0.07	-11.60 (12.44)	-37.46,14.25	0.36
Ln (Post BChE/Pre BChE) (n=29)	-14.52 (4.61)	-23.97,-5.07	0.004	-7.33(5.93)	-19.63,4.97	0.23

*Regression models adjusted for field stations, age, family income, home pesticide use and average number of hours of work in the field over the entire application season (for applicators only)

When we examined biomarker-symptom relationship by subclasses of symptoms among the applicators we observed significant positive associations of log-transformed TCPy with behavioral, autonomic, cognitive, motor and sensory problems after accounting for sociodemographic and occupational covariates (Supplementary Table 1). The magnitudes of associations (adjusted betas) were greater for autonomic, cognitive and sensory symptoms than the two other subclasses. Although the log-transformed change in AChE activity was not associated with any of these subclasses, change in BChE activity demonstrated a significant association with average percentage of behavioral symptoms ($p=0.04$) and a marginally significant association with average percentage of cognitive symptoms ($p=0.07$) (Supplementary Table 1).

DISCUSSION

A self-reported symptom questionnaire has been globally recognized as the primary method to capture symptom data in exposed populations. The most common questionnaire utilized is the extended or modified versions of Q-16²⁷, which has been used in many international studies including a study with Nicaraguans living close to cotton fields⁸, Sri Lankan farmworkers¹⁰ and Colorado agricultural communities³². However, time intervals between exposure and collection of symptom data in these studies varied from one month to twelve months^{6 9 10 28 33-35}. Furthermore, the majority of studies have utilized cross-sectional design which lacks information about temporality.

To the best of our knowledge, this is the first longitudinal study on adolescents to look into the relationship between CPF and self-reported neurological symptoms. In this study, a gradual increase in neurological symptoms relative to the baseline time interval was observed among the applicators from the 2nd to the 8th time intervals (days 24-77 of the study during June 26 to August 18, 2010) after accounting for the number of days worked during the week, home use of pesticides by the participant, age, education and family income levels. A significant 30 percentage point increase in the neurological symptoms relative to the baseline time interval was observed on the 8th time interval (days 63-77 of the study). This is perhaps due to a second short CPF application episode in the same season in the field station at Al-Shohada. Self-reported symptoms among applicators remained significantly elevated from the baseline time interval until day 217, approximately five months after the cessation of exposure showing evidence that despite discontinuation of CPF application, repeated exposure of this pesticide led to persistence of neurological health effects for several months. Compared with the applicators, the non-applicators showed relatively late reporting of neurological symptoms perhaps due to the low level environmental chlorpyrifos exposure. It is interesting to note that the non-applicators still reported approximately 9 percentage point more symptoms relative to baseline in the last time point (day 105-217). Residual CPF can survive in indoor environments for an extended period of time, can rapidly bind to soil and plants and

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3 has a half-life of several months in soil ^{36,37}. We anticipate that because of these properties, CPF remained
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5 in the environment as a potential source of environmental exposure leading to increased symptom
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7 reporting among non-applicators.
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12 The symptom reporting across time showed a recovery phase at the 10th time interval (day 105-207)
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14 when percentage of symptom reporting relative to baseline declined substantially from the previous time
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16 intervals (Table 3, Figure 1). Using the same sample, we recently demonstrated that both applicators and
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18 non-applicators experienced peak median BChE depression during the CPF application period but BChE
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20 returned to the baseline level by the end of the study (day 217/January 5, 2011) ³¹. We anticipate that
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22 symptoms were following BChE activity pattern, i.e., as the BChE activity was returning back to the
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24 baseline level, recovery from the neurological symptoms was taking place.
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30 Prior to this study, a cross-sectional study on Egyptian cotton field workers reported associations
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32 between OP exposure and neurological symptoms ^{19,20}. Similar to another Indian study on occupationally
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34 exposed adolescents ²¹, the previous Egyptian adolescent study ^{19,20} presented descriptive statistics to
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36 show the difference between exposed and unexposed adolescents in terms of the prevalence of various
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38 neurological symptoms without taking other sociodemographic confounders into account. Results of the
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40 present study were consistent with several longitudinal studies conducted in adult populations. In one
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42 study of occupationally and non-occupationally OP pesticide-exposed farmers and fishermen, delayed
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44 persistence of neurological symptoms were found during the two-year follow-up⁷. Results from a clinical
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46 examination of the same cohort found that there were deficits related to sensory function ³⁸. Another
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48 study, conducted over three years with Colorado farm workers, reported an association between OP
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50 exposure and symptoms of depression ¹². Consistency in the results across studies indicate that a Q-16
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52 based self-reported questionnaire used in all of these studies is a reliable measure to estimate health
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54 effects resulting from OP (in this case chlorpyrifos) exposure.
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6 Our study is also novel in its approach to include prospective measures of biomarkers. First, instead
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8 of using single-time point biomarker data (urinary TCPy) commonly used in cross-sectional studies, our
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10 study analyzed urinary TCPy levels at multiple time points. The collection of pre, during and post
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12 exposure samples resulting in a precise estimate of cumulative exposure from April 11 to January 5³¹.
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14 This has enabled us to overcome a historical challenge in characterizing OP exposure and allows us to
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16 subsequently examine the association of cumulative exposure with neurological symptoms. An additional
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18 limitation often encountered by past studies was the absence of established baseline AChE and BChE
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20 levels. A recent adult study examining the variation of cholinesterase levels among OP pesticides and
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22 carbamate-exposed field-workers could not establish any baseline AChE/BChE due to the mobility of the
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24 migrant study population³⁹. Another Egyptian adolescent study also reported greater reduction of
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26 acetylcholinesterase activity among the pesticide applicators compared to the controls^{19 20}. By collecting
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28 blood samples prior to the start of the application season, baseline data were established, which allowed
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30 us to compute more precise measures of change in activities of AChE and BChE from pre-exposure to
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32 post-exposure periods.
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38 Two previous studies of Kenyan and Palestinian farm workers, which measured cholinesterase levels
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40 before and after exposure, found associations between cholinesterase inhibition and respiratory, eye and
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42 neurological symptoms^{9 40}. Potential occupational confounding factors (e.g residential application of
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44 pesticides and number of days worked in agriculture into account) that are associated with neurological
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46 symptoms^{19 41} were not taken into account while examining exposure-outcome associations in these past
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48 studies. These potential confounding variables were included in our study questionnaires and later
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50 examined during statistical analysis.
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4 We identified a comparison group (non-applicators) who were similar in demographic characteristics
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6 to our applicators. It is often true that control groups in occupational settings may not be truly
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8 unexposed¹. In our study, close proximity to the agricultural field and application of pesticides at home
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10 were the two environmental factors offering some degree of OP exposure to the non-applicators as
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12 indicated by elevated urinary TCPy levels during the period of chlorpyrifos application to cotton fields³¹.
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14 To encounter this potential confounder, all statistical models were adjusted for these two variables in
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16 addition to other sociodemographic variables.
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21 It is difficult to explain why we found no relationship between TCPy and neurological symptoms
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23 among the non-applicators when a delayed effect of environmental or passive CPF exposure on symptoms
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25 was evident among this subgroup in the corresponding GEE model (Table 3 & Figure 1). One possible
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27 explanation is that the range of cumulative exposure was much lower among the non-applicators (154 to
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29 24,180 mg/g creatinine; median 2591 mg/g creatinine) compared to the applicators (232 to 28,260 mg/g
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31 creatinine; median 10318 mg/g creatinine). Small sample size and differences in cumulative exposure
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33 might have contributed to the non-significant association in the non-applicator subgroup. Some other
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35 undocumented environmental factors such as working during high temperature along with carrying a
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37 heavy backpack during CPF application might have positively confounded the association among the
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39 applicators.
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45 The non-specific nature of many of the symptoms is a limitation of the current study. In addition, the
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47 biological significance of these self-reported symptoms is unknown. However, the goal of the study was
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49 not to establish that more symptoms lead to development of any neurological disease. Rather we
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51 attempted to examine how repeated or cumulative exposure to chlorpyrifos determined the pattern of
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53 neurological symptoms over the entire season. Five of the symptoms included in our questionnaire are
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55 considered non-specific, including, headache, dizziness, fatigue, loss of consciousness and insomnia. The
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3 remaining 20 symptoms were classified into more specific neurological functions such as behavior,
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6 autonomic, sensory, cognitive or motor functions. When we excluded the five non-specific symptoms
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8 from the summary measure The estimated betas for the associations of exposure variables cumulative
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10 TCPy, change in AChE and BChE activities with average percentage of 20 neurological symptoms were
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12 3.19 ($p<0.001$), -6.11 ($p=0.60$) and -9.49 ($p=0.05$) respectively after accounting for potential covariates.
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17 Our study was conducted in an agricultural community in Egypt, which is relatively where families
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19 are primarily middle class to lower middle class. Results of our study may be generalizable only to
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21 agricultural communities with similar sociodemographic characteristics.
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24 25 CONCLUSION

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27 Our study is the first to demonstrate that repeated occupational CPF exposure is an important
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29 determinant of neurological symptoms in adolescent applicators and non-applicators across time, with
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31 symptoms peaking during the exposure season and partly recovering in months following exposure. The
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33 study also showed significant association between cumulative CPF exposure and symptoms, using
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35 cumulative urinary TCPy as a biomarker of exposure. Future studies are needed to assess the temporal
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37 and dose-dependent effects of repeated CPF exposure on neurological symptoms and neurobehavioral
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39 deficits in children, adolescents and adults to identify the most sensitive populations. Similar prospective
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41 studies with a larger population are also needed to assess the relationship between these endpoints and
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43 biomarkers of exposure, effect and susceptibility, ultimately identifying biomarkers, which may help
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45 protect sensitive population.
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5
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7
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9
10 University.

11 **Contributorship Statement**

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14 All authors have made substantial contributions to conception and design, acquisition of data, or analysis
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16 and interpretation of data in the study; Dr. Khalid Khan have taken the lead to draft the manuscript.

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18 Whereas other authors have revised the draft critically for important intellectual content; All of the
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20 authors have provided final approval of the version to be published. Dr. Diane Rohlman is the Principal
21
22 Investigator of the study and has supervised each step of the manuscript development process. She has
23
24 been listed as the Corresponding author.

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33 **IRB Approval:**

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36 The study was approved by the OHSU IRB in June 2009 and by the Medical Ethics committee of the
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38 Faculty of Medicine, Menoufia University in July 2009.

39 **Conflict of Interest Statement**

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42 None of the authors has any potential financial, personal or other conflict of interest, which could
43
44 inappropriately influence this study.

Data Sharing Statement

Additional unpublished data from the study are available to Dr. Diane Rohlman (diane-rohlman@uiowa.edu), Dr. Ahmed Ismail (aa-ismail@hotmail.com) and Dr. James Olson (aa-ismail@hotmail.com) in Excel or SPSS datasets. They can be reached by email.

Abbreviations: CPF (chlorpyrifos); OP (organophosphorus); TCPy (3,5,6-trichloro-2-pyridinol); AChE (acetylcholinesterase); BChE (butyrylcholinesterase); CYP (cytochrome P450)

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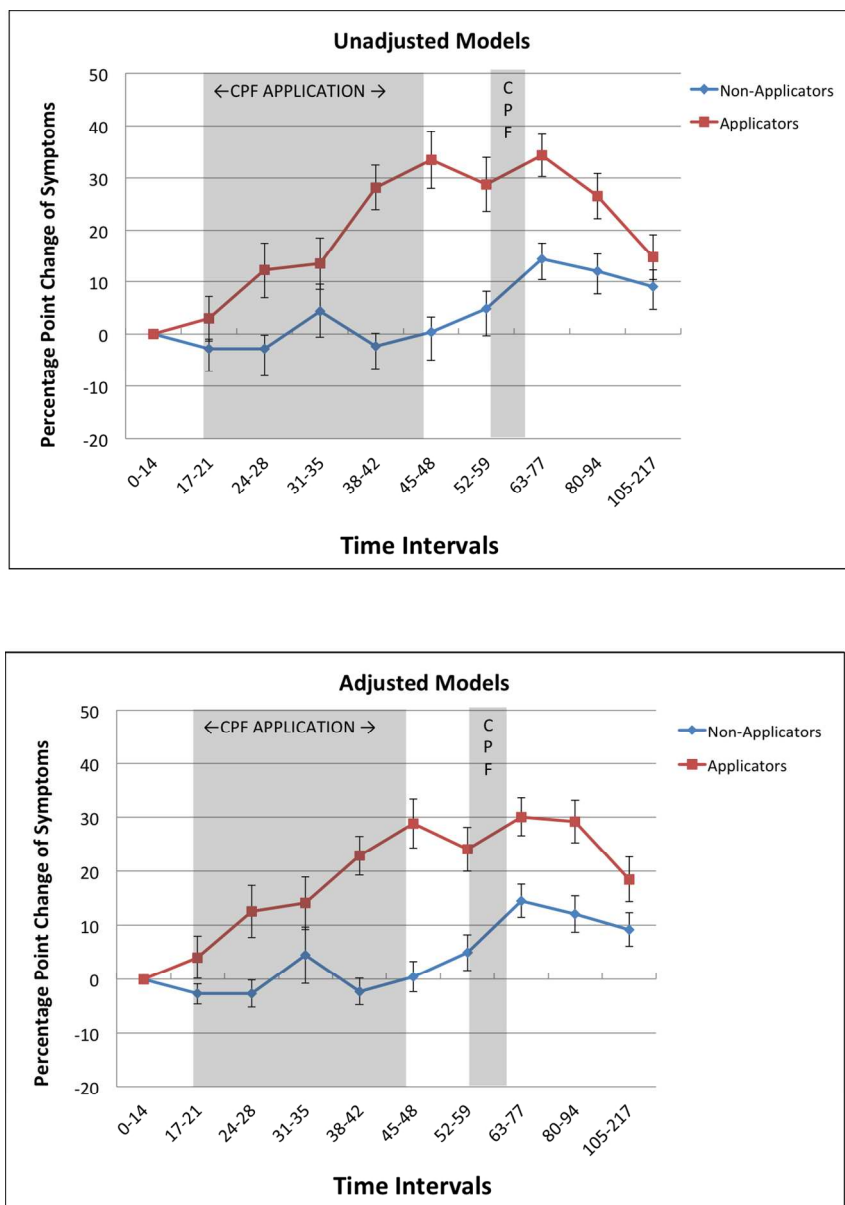


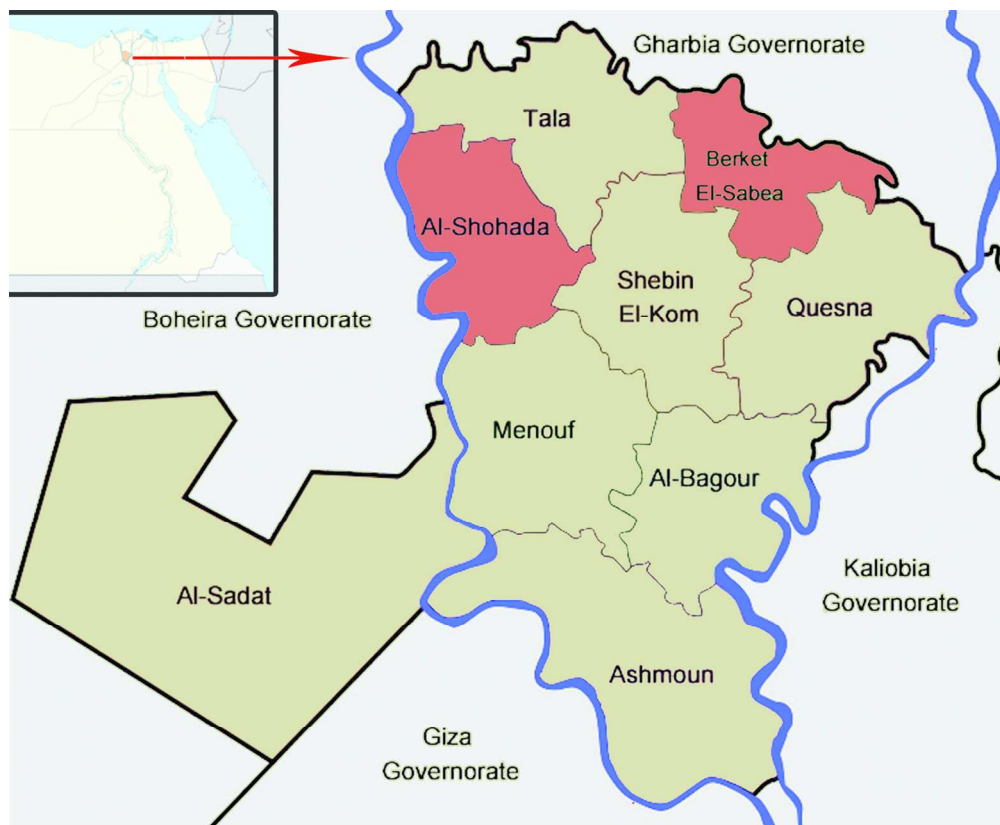
Figure 1. Difference, relative to baseline, in the percentage of symptoms reported at each of nine subsequent time intervals; error bars represent 95% confidence limits for the difference.

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3 Supplementary Figure 1. Map of Menoufia governorate showing the study districts
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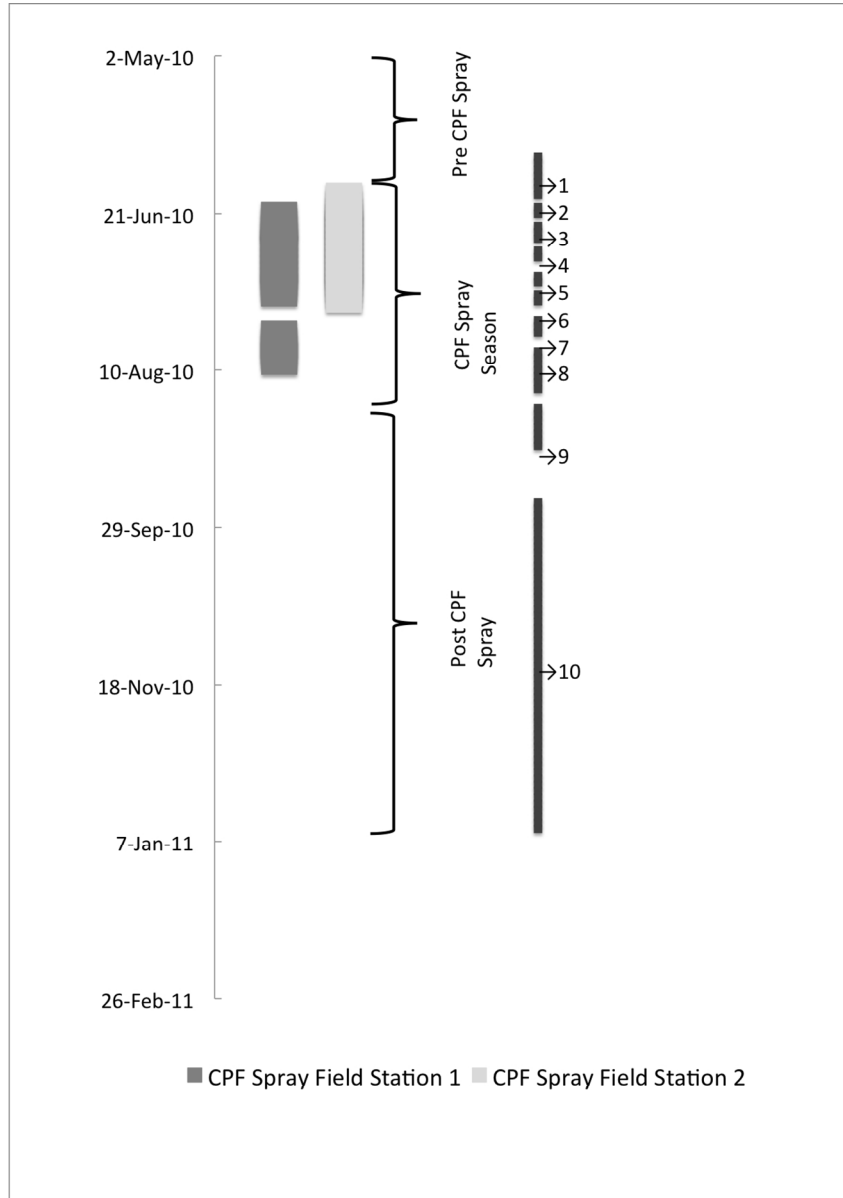
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9 Supplementary Figure 2. CPF application in the study area showing time intervals. Field stations 1 and 2
10 were located in Al-Shohada and Berket El-Sabea respectively.
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16 Supplementary Figure 3. Scatter plots of (a) cumulative TCPy (ug/g creatinine) against percentage of
17 symptoms (n=70), (b) log-transformed post AChE/pre AChE against percentage of symptoms (n=49) and
18 (c) log-transformed post BChE/pre BChE against percentage of symptoms (n=50)
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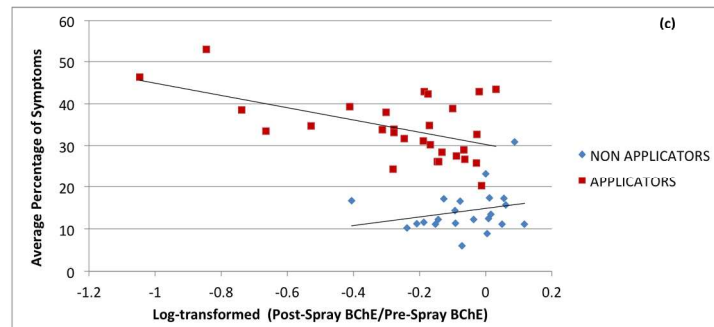
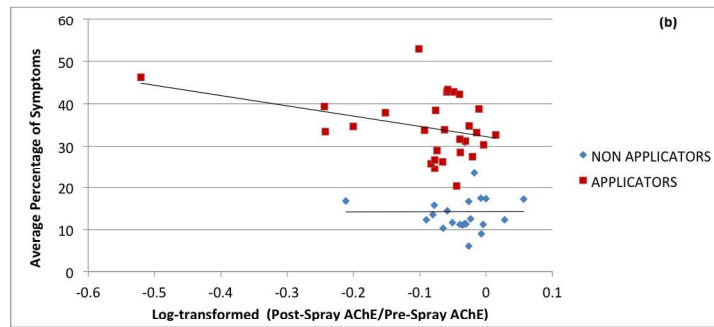
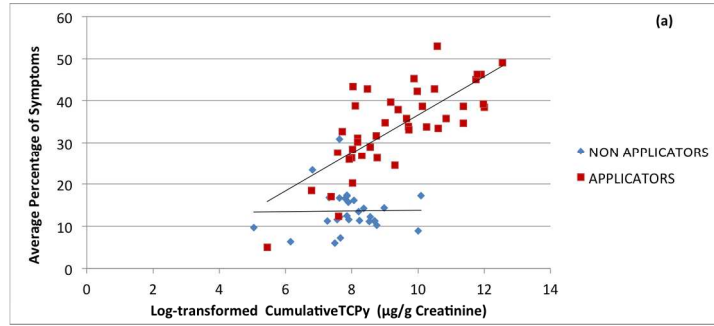


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Supplementary Table 1. Summary of regression analysis for biomarkers of exposure and effect of chlorpyrifos predicting percentage of subclasses neurological symptoms among Applicators

Outcome variable (Symptom Subclass)	Unadjusted Model		Adjusted Model		Unadjusted Model		Adjusted Model		Unadjusted Model		Adjusted Model	
	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value
Behavior & Affect	2.16 (0.57)	<0.001	2.80 (0.91)	0.004	-12.59 (10.86)	0.26	-16.34 (16.04)	0.33	-6.96 (4.09)	0.10	-12.26 (5.73)	0.04
Autonomic	5.69 (0.75)	<0.001	3.24 (0.99)	0.002	-38.45 (14.88)	0.02	-4.84 (17.22)	0.78	-19.65 (5.42)	0.001	-9.69 (6.40)	0.14
Cognitive	6.92 (0.87)	<0.001	5.27 (1.34)	<0.001	-46.22 (17.49)	0.01	1.73 (20.82)	0.94	-24.16 (6.03)	0.001	-13.56 (7.55)	0.07
Motor	2.48 (0.49)	<0.001	1.67 (0.74)	0.03	-6.27 (8.41)	0.46	4.50 (12.03)	0.37	-5.75 (3.15)	0.08	-3.90 (4.64)	0.41
Sensory	6.47 (0.90)	<0.001	4.94 (1.34)	0.001	-27.49 (17.64)	0.13	-0.75 (22.46)	0.97	-20.00 (6.41)	0.004	-13.88 (8.86)	0.13
Temporary Disability (Non-specific)	5.48 (0.61)	<0.001	3.64 (0.78)	<0.001	-41.13 (14.14)	0.007	-10.74 (16.61)	0.53	-21.80 (4.73)	0.001	-13.52 (5.73)	0.03

*Regression models adjusted for field stations, age, family income, home pesticide use and average number of hours of work in the field over the entire application season (for applicators only)

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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract: Title indicates "Longitudinal Assessment....." (b) Provide in the abstract an informative and balanced summary of what was done and what was found: Summary provided in the abstract
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported: Explained
Objectives	3	State specific objectives, including any prespecified hypotheses: Objectives specified
Methods		
Study design	4	Present key elements of study design early in the paper: Presented in the last paragraph of the Introduction section
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection: Described in the Method section
Participants	6	(a) <i>Cohort study</i> —Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up: Described in the Method section (b) <i>Cohort study</i> — This is not a matched study; Convenience sampling
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable: Described in Methods
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group: Provided
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at: Explained in Method
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why: Described
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding: Described in statistical analysis section (b) Describe any methods used to examine subgroups and interactions: Described (c) Explain how missing data were addressed: No missing data (d) <i>Cohort study</i> — Not applicable (e) Describe any sensitivity analyses- Not applicable

Continued on next page

Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed: Reported (b) Give reasons for non-participation at each stage: Not applicable (c) Consider use of a flow diagram: Not required as a figure in the result section explained the time-intervals when the participants were observed for the outcome
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders: Provided (b) Indicate number of participants with missing data for each variable of interest: Not applicable (c) <i>Cohort study</i> —Summarise follow-up time (eg, average and total amount): Summarized
Outcome data	15*	<i>Cohort study</i> —Report numbers of outcome events or summary measures over time: Reported
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included: Clear explanation of the estimates are provided (b) Report category boundaries when continuous variables were categorized: Not applicable (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period: Not relevant
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses: Not performed and therefore not reported
Discussion		
Key results	18	Summarise key results with reference to study objectives: Summary of key findings are presented in the Discussion section
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias: Limitations discussed in the last paragraph of the Discussion section
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence: Provided in the Discussion section
Generalisability	21	Discuss the generalisability (external validity) of the study results: Discussed in the paper
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based: Provided after the Discussion section just before the list of references

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies: **Not applicable**

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.



Longitudinal Assessment of Chlorpyrifos Exposure and Self-Reported Neurological Symptoms in Adolescent Pesticide Applicators

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Primary Subject Heading:	Occupational and environmental medicine
Secondary Subject Heading:	Global health, Neurology, Public health
Keywords:	Public health < INFECTIOUS DISEASES, Toxicology < PATHOLOGY, Epidemiology < ONCOLOGY

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Manuscripts

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4 **Longitudinal Assessment of Chlorpyrifos Exposure and Self-Reported Neurological**
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6 **Symptoms in Adolescent Pesticide Applicators**
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Key Words: chlorpyrifos, neurological symptoms, TCPy, cholinesterase, occupational exposure

ABSTRACT

Objectives: Occupational exposure of organophosphorus pesticides (OPs), such as chlorpyrifos (CPF), in adolescents is of particular concern because of the potential vulnerability of the developing neurological system. The objectives of the study were to examine how neurological symptoms reported over the CPF application season vary across time, whether these effects are reversible post application and if there are any associations between CPF biomarkers and neurological symptoms in an adolescent study population.

Methods: Egyptian adolescent CPF applicators (n=57) and non-applicators (n=38) were recruited for a longitudinal study. Self-reported data for 25 neurological symptoms were collected at 32 time points over the 8-month period before, during and after CPF-application. Urine and blood samples were collected for CPF-specific biomarkers: urine trichloro-2-pyridinol (TCPy) and blood cholinesterase.

Results: When we compared reporting of symptoms between applicators and non-applicators at different time intervals over the 8-month study period, we observed both groups reporting the highest numbers of symptoms in the middle of the CPF application season. Applicators reported a greater percentage of neurological symptoms, relative to baseline, than the non-applicators after accounting for potential covariates. Only among the applicators, cumulative TCPy was positively and significantly associated with the average percentage of symptoms. Significant

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3 associations between the change in butyrylcholinesterase (BChE) from pre to post application
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5 season and several domains of neurological symptoms were also found even after adjusting for
6
7 potential covariates.
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10 **Conclusions:** These observations reinforce the growing concern regarding the neurotoxic health
11 effects of CPF in adolescent applicators in developing countries and the need for developing and
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13 implementing intervention programs through increased use of personal protective equipment.
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28 **STRENGTHS AND LIMITATIONS OF THE STUDY:**

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- 32 • This is the first longitudinal study demonstrating an association between CPF exposure
33 and reporting of neurological symptoms in adolescent applicators.
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 - 35 • The study is also novel in its approach to include prospective measures of biomarkers of
36 CPF exposure and effect and to examine their associations with neurological symptoms.
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 - 38 • The non-specific nature of many of the symptoms is a limitation of the current study.
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 - 40 • Small sample size of this study may have influenced the significance levels of exposure-
41 outcome relationships.
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What this paper adds

- Applicators are more likely to report increased symptoms compared to non-applicators.
- Repeated occupational exposure to CPF increases the reporting of acute neurological symptoms during the CPF application season; the symptoms persist for months after the cessation of exposure in both applicators and non-applicators.
- Cumulative biomarker of CPF exposure (TCPy) also demonstrates an association with neurological symptoms in applicators.
- Reduction of CPF exposure among the adolescent applicators and non-applicator residents of agricultural communities should be a public health priority since neurological symptoms remained elevated even after the cessation of CPF application.

INTRODUCTION

The high use of organophosphorus pesticides (OPs) has been recognized as a major global public health challenge for agriculture-based communities, due to their associations with adverse neurological outcomes. Immediate and short-term neurological signs and symptoms ranging from less severe (headache, dizziness, nausea etc.) to more severe (muscle weakness, bronchospasm, change in heart rate etc.) have all been reported after occupational exposure to OPs^{1 2}. Although high levels of occupational OP exposure can be associated with symptoms persisting for several years³, repeated, moderate to low exposures, can also produce chronic neurological symptoms and deficits in neurobehavioral performance⁴. Converging evidence regarding the associations between OP exposures and neurological symptoms is based on occupational studies with adults conducted in a wide range of settings; including comparisons between exposed and non-exposed farmworkers in the US⁵, South Africa⁶, Nicaragua^{7 8}, Kenya⁹, Sri Lanka¹⁰ and Egypt¹¹. Additional evidence for the effect of pesticides on somatic and mood symptoms are also found in the literature^{2 12}.

Although it is illegal there have reports of involvement of US adolescents in mixing and applying pesticides in some agricultural communities^{13 14}. The developing bodies of children and adolescents may not break down pesticide as effectively as adult and they may receive a larger dose per unit of body weight for a given exposure due to their smaller body size¹⁵, making them more vulnerable to neurological effects. Animal and human studies have also suggested that paraoxonase PON-1, an organophosphate detoxifying enzyme, is less active in younger populations making them more vulnerable to OP toxicity^{16 17}. A recent study has found association of environmental CPF exposure with structural changes in developing brain of the

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3 children and adolescents¹⁸. In developing countries, children and adolescents are engaged in
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5 risky agricultural activities including the application of OPs¹⁹. In two epidemiological studies,
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8 Egyptian and Indian children and adolescents living in agricultural communities have
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10 demonstrated associations between occupational and environmental OP exposure and
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12 neurological and neuromuscular problems²⁰⁻²².

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18 Biomarkers have been used to characterize OP exposure in epidemiological and
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20 occupational studies. Urinary trichloro-2-pyridinol (TCPy) is a relatively specific metabolite of
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22 CPF exposure, eliminated in the urine with a half-life of 27 hours following exposure²³. Due to
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24 the ease and non-invasiveness of the collection of urine samples, TCPy is widely recognized as a
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26 useful biomarker of exposure, particularly in children and adolescents^{24 25}. The classic mode of
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28 OP toxicity is manifested by the inhibition of cholinesterase. Both blood acetylcholinesterase
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30 (AChE) and butyrylcholinesterase (BChE) are biomarkers of effect, with BChE being more
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32 sensitive to inhibition by OP pesticides²⁶. A small number of adult studies found associations
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34 between inhibition of cholinergic activities with self-reported symptoms^{9 10}; however, this
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36 relationship has rarely been examined in adolescent studies.
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44 Understanding the relationship between OP exposure and the change in neurological
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46 symptoms over time (temporal change) is important because application-related exposure
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48 follows a seasonal pattern in most areas. Two longitudinal studies with agricultural workers
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50 demonstrated that short-term neurological signs and symptoms were associated with initial acute
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52 episodes of exposure, which eventually advanced into long-term sequelae^{7 12}. However, these
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3 studies did not characterize exposure by identifying specific types of OPs that were related to the
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5 symptoms.
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10 The primary objective of this study was to determine whether occupational exposure to CPF
11 is associated with self-reported neurological symptoms in adolescents. Through a prospective
12 study, we compared adolescent applicators exposed to CPF with adolescent non-applicators
13 working and residing in agricultural communities in Egypt. Typically, CPF is the primary
14 insecticide used by pesticide applicators in Egyptian cotton fields; offering us a unique exposure
15 opportunity with well characterized occupational exposure. The possibility of potential
16 confounding effects of other neurotoxic pesticides was minimal because of limited use of other
17 pesticides in the study area. The goals of the study were to examine how neurological symptoms
18 vary over time during the exposure season, if these effects could reverse at the cessation of
19 exposure and whether there are any associations between CPF biomarkers and neurological
20 symptoms in the adolescent study population.
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39 **METHODS**

40 **Study area and population**

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42 Two agricultural districts were selected from Menoufia Governorate, Egypt
43 (Supplementary Figure 1) to conduct a prospective study from April 2010 to January 2011. In
44 Egypt, adolescents are hired seasonally to apply pesticides to cotton fields and the schedule of
45 pesticide applications to the cotton crop is regulated by the Ministry of Agriculture. The typical
46 workday was from 8am-12pm and from 3pm-7pm, six days per week. During 2010,
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48 approximately 2100 liters of OPs were applied to 5700 acres of cotton fields (personal
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4 communication with the Ministry of Agriculture). Chlorpyrifos is the primary OP applied to the
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6 cotton crop from mid-June to early August. Although there are slight variations in the timing of
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8 CPF application between the two districts (Figure 1), the application patterns are consistent
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10 across these two areas. Because there is no regulation in Egypt for mandatory use of personal
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12 protective equipment (PPE), dermal exposure and inhalation were both considered as the
13
14 potential route of exposure in this population²⁰. Recently, Fenske et al.²⁷ reported that dermal
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16 exposure and subsequent absorption through the skin accounted for 94-96% of the total dose of
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18 CPF in Egyptian pesticide applicators.
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22 23 24 25 **Recruitment and data collection**

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27 Fifty-eight male adolescents aged 12-21 that were hired seasonally by the Ministry of
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29 Agriculture to spray pesticides in the cotton fields, were recruited from two districts in the
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31 Menoufia governate. Forty adolescent non-applicators were recruited through convenience
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33 sampling (i.e., word of mouth, direct communication utilizing contacts through the staff from the
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35 local Ministry of Agriculture) from the same districts as the applicators for the cotton crop.
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37 These adolescents never worked in the cotton fields as pesticide applicators. One adolescent was
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39 excluded from the final analysis due to his inconsistency in participating in the study activities
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41 and two other participants were excluded for questionable sample integrity, resulting in a final
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43 sample size of 95 (57 applicators and 38 non-applicators). Written informed consent was
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45 obtained from all participants and their legal guardian (for those under 18). All the subjects were
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47 monetarily compensated for their time during the questionnaire survey and biological samples
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49 (~\$5 per visit). The study was approved by the Oregon Health and Science University IRB in
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3 June 2009, and by the Medical Ethics committee of the Faculty of Medicine, Menoufia
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6 University in July 2009.
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8 Data collection, for both applicators and non-applicators, occurred at the primary field
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10 station for each district. Pesticides applicators and supervisors meet in the field stations, which
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12 also provides storage area for the pesticides and the equipment used for application.
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15 16 17 18 **Outcome assessment**

19 We developed a 25-item, short-term neurological symptom questionnaire on the basis of
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21 the widely used Q16 questionnaire²⁸ and a modified version of the Q16 used in a previous study
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23 on licensed pesticide applicators²⁹. The 25 symptoms were grouped into six domains:
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25 behavioral, autonomic, cognitive, sensory, motor and non-specific temporary disability (Table
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27 1). The questionnaire had five response options (0-4) for each symptom ranging from “never”
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29 (coded as 0) to “everyday of the week” (coded as 4). Since more than 90% of the responses were
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31 between 0-2 (1=once a week and 2=once in every 2-3 days) we recoded each of the symptom
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33 response to “0” or “never” and “1” or “at least once a week or more.” Self-reported neurological
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35 symptom counts were collected at 32 irregularly spaced dates over an eight-month period from
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37 early June 2010 through early January 2011. These time points ranged across three different time
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39 periods: pre-application, application, and post-application. For each time point, the number of
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41 positive responses (a response was considered positive and coded as “1” when the participant
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43 reported the frequency of the symptom “at least once a week or more”) was totaled for each
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45 person to yield a score ranging from 0–25; division by 25 produced the proportion of symptoms
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47 endorsed at each of the 32 time points. This outcome variable was used to compare the change
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49 of symptoms over time between applicators and non-applicators. All these time points were
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4 collapsed into 10 separate non-overlapping intervals lasting between one and four weeks in
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6 length (Figure 1). Symptom data during the pre-application period, including the first fifteen
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8 days of the study was collectively taken to represent the baseline time interval (or time interval
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10 1). Symptom reporting from the other nine remaining time intervals was evaluated against time
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12 interval 1. The next 5 time intervals, between June 19 and July 21, were during the application
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14 period of CPF. The remaining 4 time intervals occurred between July 24, 2010 and January 5,
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16 2011 and reflect the post-application period although a brief CPF application was reported in the
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18 district where field station 1 was located. The proportions of symptoms over all the 32 time
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20 points were averaged to produce a season-level average percentage of neurological symptoms
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22 over the entire study period. This outcome variable was used to examine the relationships
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24 between the biomarkers (TCPy, AChE and BChE) and symptoms. Participants also completed a
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26 questionnaire during baseline addressing their sociodemographic status, household and
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28 occupational use of pesticides.
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Table 1. Domains of neurological symptoms

Domains	Symptoms
Behavioral Symptoms	1. Tense or anxious [#] 2. Excessively angry or irritable ^{*#} 3. Depressed or withdrawn ^{*#}
Autonomic Symptoms	4. Nausea [#] 5. Heavy sweating ^{*#} 6. Loss of appetite [#] 7. Fast heart rate ^{*#} 8. Excessive salivation
Cognitive Symptoms	9. Difficulty concentrating ^{*#} 10. Being absentminded and memory problem ^{*#}
Sensory Symptoms	11. Difficulty seeing at night [#] 12. Blurred or double vision [#] 13. Numbness in hands and feet [#] 14. Sense of smell or taste change [#] 15. Ringing in ears
Motor Symptoms	16. Difficulty with balance [#] 17. Weakness in arms and legs [#] 18. Involuntary movement of arms and legs [#] 19. Shaking in hands [*] 20. Difficulty speaking [#]
Temporary Disability (non-specific symptoms)	21. Dizziness [#] 22. Headache ^{*#} 23. Momentary loss of consciousness [#] 24. Fatigue ^{*#} 25. Insomnia [#]

*Symptoms used in Q16²⁷

#Symptoms used in Agricultural Health Study²⁸

Urine collection and analysis

Spot urine samples were collected in new and individually wrapped cups at the beginning of the work shift at eight time points between April 2010 and January 2011. The cups were opened at the time of sample collection. Urine samples were subsequently transferred to the laboratory at Menoufia University in a cooler with wet ice. At the laboratory, 4 ml aliquots of urine were transferred into labeled 5 ml cryovials within hours of sampling and stored at -20°C . The banked urine samples were express mailed on dry ice to the University of Buffalo laboratory for analysis of pesticide metabolites; duplicate samples were retained in the -20°C freezer at Menoufia University. Urine samples in the field station 2 were collected one day after the collection date of the field station 1.

Creatinine concentrations were measured using the Jaffe reaction³⁰. The method of urinary TCPy measurement (a primary metabolite of chlorpyrifos) has been described elsewhere²⁴. Briefly, Samples were analyzed using gas chromatography–mass spectrometry (negative-ion chemical ionization) and utilized ^{13}C - ^{15}N -3,5,6-TCPy as an internal standard. Samples were hydrolyzed with HCl, extracted with toluene, and derivatized using N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide (Sigma Aldrich, USA). A spiked quality control (QC) sample was routinely run with the analytical samples and the metabolite concentration was determined from a standard curve for the peak area for the selective ion. The QC samples consisted of lab samples that were first analyzed for TCPy and the levels were non-detectable. The TCPy standard curve was linear from 1-200 ng/ml with a correlation coefficient of 1.000. Samples spiked with 50ng of TCPy/mL (n=20) gave an average metabolite recovery of 94.8% (range = 92 - 98%; SD = 0.931;

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4 RSD% = 1.965). A 1ng TCPy/ml spiked sample was run 10 times and the within series RSD% =
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6 1.6. The minimum detection level was 0.5 ng/ml of urine.
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10 **Blood collection and ChE analysis**

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12 To establish the baseline ChE activity, pre-application blood draws occurred on April 11
13 and June 2, 2010, prior to the start of the official government-regulated CPF application season.
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15 As with the urine collection, blood draws occurred in the field station 2 one day later. Changes in
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17 both AChE and BChE levels from baseline to the end of CPF-application season (blood collected
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19 on September 4, 2010) were estimated. Blood samples were collected by venipuncture into
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21 10mL lavender top (EDTA) vacutainer tubes and immediately placed on wet ice and transported
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23 to Menoufia University, where they were analyzed in duplicate for AChE and BChE activity
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25 using an EQM Test-Mate kit (EQM Research Inc., Cincinnati, OH, USA) as described
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27 previously²⁴.
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37 **Statistical analysis**

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39 We used SPSS version 18.0 and STATA (version 11; Stata Corporation, College Station,
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41 TX) for the statistical analysis. Sociodemographic variables were summarized and described
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43 using means and standard deviations for continuous responses and percentages for discrete
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45 outcomes; simple comparisons between applicators and non-applicators were completed using t-
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47 tests or chi-square tests. To calculate the value of cumulative TCPy for each participant we used
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49 STATA's pharmacokinetic function (pkexamine) to employing the trapezoid rule to estimate the
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51 area under the curve for each participant over the study time. By definition, cumulative TCPy
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53 was the sum of the concentration at each time point multiplied by the duration between time
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4 points. This variable reflects the total amount of TCPy excreted over the study period for which
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6 urine as collected and assayed. Concentrations of cumulative TCPy, AChE and BChE exhibited
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8 pronounced right skewed distribution and more than a 3-fold separation between the minimum
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10 and maximum observed values; consequently, these responses were log-transformed prior to
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12 analysis to improve symmetry. Both AChE and BChE were expressed as a log-transformed ratio
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14 of post-application activity relative to pre-application activity. Then the associations between the
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16 change of these cholinesterase markers from pre to post application seasons and self-reported
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18 symptoms were examined using linear regression models that took potential covariates into
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20 account. Similar regression analyses were used to examine the relationship between cumulative
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22 TCPy and neurological symptoms. All p-values are two-sided with significance judged relative
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24 to a 0.05 level.
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32 Spearman correlation coefficients were used to estimate associations between urine and
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34 blood biomarkers and symptom scores. Generalized estimating equations (GEE)³¹ were used to
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36 model the proportion of neurological symptoms reported in each time interval while controlling
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38 for number of days worked (within five days of the symptom reporting date), home use of
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40 pesticides, age, education and income levels. The one fitted model was used to estimate changes
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42 over time, relative to the first time interval (June 2–June 16), for applicators and non-applicators,
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44 as well as to examine whether changes relative to baseline differed between the two groups (via
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46 group-by-time interaction).
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RESULTS

Sociodemographic Characteristics

Ninety-two of the participants (97%) were between 12 and 18 years old with the remaining three between 19 and 21. The two groups, non-applicators and applicators, did not differ significantly in terms of age, educational status, family income, number of people in house, years of pesticide use at home, and insecticides and rodenticides use at home (Table 2). Compared to non-applicators, a significantly higher number of applicators lived close to the field (within 25 meters), had carpet in their homes and applied herbicides at home. Applicators had significantly lower BMI than non-applicators. On average, applicators had been working in the field for a little over 3 years.

Table 2: Sociodemographic characteristics for participants at baseline

Variables	Non-applicators (n=38)	Applicators (n=57)
	Mean (SD)	Mean (SD)
Age	16.6 (2.4)	16.2 (1.6)
Education	9.8 (1.8)	9.9 (1.8)
Height (cm) #	166.3 (12.0)	163.4 (10.0)
Weight (kg)*	62.0 (15.4)	54.2 (8.6)
BMI (kg/m ²)*	22.1 (3.7)	20.2 (2.2)
Number of people in house	5.6 (1.1)	6.0 (1.8)
Home pesticide use (years) ^{##*}	1.6 (1.9)	2.5 (1.9)
Occupational application of pesticides (yrs)	-	3.1 (1.5)
Days/week of pesticide application	-	4.8 (1.3)
Hours/day of pesticide application	-	5.2 (0.7)
	% (n)	% (n)
Family Monthly Income (<500 E)	78.9 (30)	71.9 (41)
Applied pesticides in home in last 5 yrs (yes)*	47.4 (18)	78.9 (45)
Computer use (once a week or more)*	65.8 (25)	45.6 (26)
Carpet in house (yes)*	27.0 (10)	54.4 (31)
Live within 25m to agricultural field (yes)*	23.7 (9)	50.9 (29)
Types of pesticides applied at home ^{###}		
Herbicides*	13.0 (3)	44.9 (21)
Insecticides	83.3 (20)	93.9 (46)
Rodenticides	16.7 (4)	14.3 (7)

*p<0.05 for group difference; #30 applicators vs 34 non applicators, ##44 applicators vs 19 non applicators, #49 applicators vs 24 non applicators, E=Egyptian pound

Change in symptoms over time

We considered days 0-14 as the baseline time interval (time interval 1) when no application of CPF was reported. While examining symptoms reported over time among both applicators and non-applicators we took various potential confounders into account. These include occupational factors such as days worked per week in pesticide applications, number of years of pesticide use at home and socio-demographic factors such as age, education and income level of the participants. Applicators began increased reporting of neurological symptoms at the beginning of the CPF application season (at time interval 2 between days 17-21 of the study). The percentage of neurological symptoms continued to increase during the application season and reached the peak at time interval 6, representing days 45-48, the time when CPF application period ended. This was followed by a drop of symptom reporting indicating a small recovery due to the cessation of exposure in both districts. The highest peak of symptom reporting was observed at the time interval 8 representing days 63-77 (Table 3). This happened perhaps due to a small episode of CPF application in field station 1 (between time intervals 7 and 8). Similar to the applicators, the non-applicators also demonstrated the highest increase in the proportion of neurological symptoms during the time interval 8 although the magnitude of the change was smaller (14 percentage point increase of symptoms relative to baseline interval). The change of neurological symptoms relative to baseline declined over the next two time intervals (9 and 10) in both groups indicating a recovery phase during post-application. For applicators, the percentage of reported symptoms at each of the nine subsequent time intervals was always higher than the percentage observed at baseline; non-applicators, by contrast, had a pattern of reported symptoms that both increased and decreased relative to the baseline time interval (pre-application) over the course of the study (Figure 2).

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6 When applicators and non-applicators are compared with respect to change in percentage
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8 of symptoms (relative to baseline), it was always the case that the change (percentage point
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10 change relative to baseline) for applicators was greater than the corresponding change for non-
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12 applicators even after adjusting for the covariates (Table 3).
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Associations of neurological symptoms with biomarkers

TCPy was detected in 100% of the samples. Summary statistics for TCPy, AChE and BChE of the study samples have been already reported by Crane et al. (2013)³², Mean creatinine concentration of the urine samples was reported to be 1696 μ g/ml with a maximum of 4199 and a minimum of 164 μ g/ml. In brief, the applicators had much higher mean and estimated median peak TCPy concentration than the non-applicators (mean: 719 vs 44.9 μ g/g creatinine; estimated median 137 vs 19.7 μ g/g creatinine). In our study sample, BChE was found to be more sensitive to CPF exposure than AChE, with median activity reduced by 37% from baseline in applicators and 13% in non-applicators during the CPF application period.

A scatter plot of cumulative TCPy (ug/g creatinine) against average percentage points of symptoms revealed distinct exposure-response gradients by pesticide application status (applicators vs non-applicators) (Figure 3). In addition, two other scatter plots of change in AChE activity and change in BChE activity from pre-application to post-application against percentage of symptoms also revealed effect measure modification by pesticide application status (Supplementary Figures 2a & 2b). Therefore, separate linear models for applicators and non-applicators were used to examine the associations of these three biomarkers with symptoms.

Log-transformed TCPy was positively associated with the average percentage of neurological symptoms in the regression models after adjusting for other covariates that may confound exposure-outcome relationship such as field stations, age, family monthly income, pesticide use at home and average number of hours worked in the field among applicators (b=2.68, p=0.007). However, non-applicators demonstrated positive, but statistically non-significant, associations between TCPy and symptoms. Among applicators, AChE and BChE

activity was negatively and significantly associated with the average percentage of neurological symptoms in the unadjusted models. In the adjusted models these associations remained negative but became non-significant (Table 4).

Table 4. Summary of regression analysis for biomarkers of exposure & effect of chlorpyrifos predicting average percentage of neurological symptoms over the entire study stratified by applicator status

Explanatory variables	Unadjusted Models			Adjusted Models*		
	B (se)	95% CI	p-value	B (se)	95% CI	p-value
<i>For Non Applicators</i>						
Ln TCPy (mg/g Cr) (n=28)	0.29 (0.76)	-1.26,1.84	0.71	0.57 (0.79)	-1.06,2.20	0.47
Ln (Post AChE/Pre AChE) (n=21)	-1.25 (16.41)	-35.59,33.1	0.94	-6.57 (18.80)	-43.64,33.50	0.73
Ln (Post BChE/Pre BChE) (n=21)	2.23 (7.04)	-12.51,16.98	0.76	2.50 (7.63)	-13.77,18.77	0.75
<i>For Applicators</i>						
Ln TCPy (mg/g Cr) (n=42)	4.56 (0.63)	3.29,5.84	<0.001	2.68 (0.93)	0.78,4.57	0.007
Ln (Post AChE/Pre AChE) (n=28)	-24.21 (12.79)	-50.50,2.09	0.07	-11.60 (12.44)	-37.46,14.25	0.36
Ln (Post BChE/Pre BChE) (n=29)	-14.52 (4.61)	-23.97,-5.07	0.004	-7.33(5.93)	-19.63,4.97	0.23

*Regression models adjusted for field stations, age, family monthly income, pesticide use at home and average number of hours of work in the field over the entire application season (for applicators only)

When we examined the biomarker-symptom relationship by domains of symptoms among the applicators, we observed significant positive associations of log-transformed TCPy with behavioral, autonomic, cognitive, motor and sensory problems after accounting for sociodemographic and occupational covariates (Table 5). The magnitudes of associations (adjusted betas) were greater for autonomic, cognitive and sensory symptoms than the two other domains. Although the log-transformed change in AChE activity was not associated with any of these subclasses, change in BChE activity demonstrated a significant association with average

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3 percentage of behavioral symptoms ($p=0.04$) and a marginally significant association with
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5 average percentage of cognitive symptoms ($p=0.07$)
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Table 5. Summary of regression analysis for biomarkers of exposure and effect of chlorpyrifos predicting average percentage of neurological symptoms by subclasses among **Applicators**

Outcome variable (Symptom Subclass)	Unadjusted Model		Adjusted Model		Unadjusted Model		Adjusted Model		Unadjusted Model		Adjusted Model	
	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value
Behavior & Affect	2.16 (0.57)	<0.001	2.80 (0.91)	0.004	-12.59 (10.86)	0.26	-16.34 (16.04)	0.33	-6.96 (4.09)	0.10	-12.26 (5.73)	0.04
Autonomic	5.69 (0.75)	<0.001	3.24 (0.99)	0.002	-38.45 (14.88)	0.02	-4.84 (17.22)	0.78	-19.65 (5.42)	0.001	-9.69 (6.40)	0.14
Cognitive	6.92 (0.87)	<0.001	5.27 (1.34)	<0.001	-46.22 (17.49)	0.01	1.73 (20.82)	0.94	-24.16 (6.03)	0.001	-13.56 (7.55)	0.07
Motor	2.48 (0.49)	<0.001	1.67 (0.74)	0.03	-6.27 (8.41)	0.46	4.50 (12.03)	0.37	-5.75 (3.15)	0.08	-3.90 (4.64)	0.41
Sensory	6.47 (0.90)	<0.001	4.94 (1.34)	0.001	-27.49 (17.64)	0.13	-0.75 (22.46)	0.97	-20.00 (6.41)	0.004	-13.88 (8.86)	0.13
Temporary Disability (Non-specific)	5.48 (0.61)	<0.001	3.64 (0.78)	<0.001	-41.13 (14.14)	0.007	-10.74 (16.61)	0.53	-21.80 (4.73)	0.001	-13.52 (5.73)	0.03

*Regression models adjusted for field stations, age, family monthly income, pesticide use at home and average number of hours of work in the field over the entire application season (for applicators only)

DISCUSSION

The self-reported symptom questionnaire has been globally recognized as the primary method to capture symptom data in exposed populations. The most common questionnaire utilized is the extended or modified versions of Q16²⁸, which has been used in many international studies including a study with Nicaraguans living close to cotton fields⁸, Sri Lankan farmworkers¹⁰ and Colorado agricultural communities³³. However, time intervals between exposure and collection of symptom data in these studies varied from one month to twelve months^{6 9 10 29 34-36}. Furthermore, the majority of studies have utilized cross-sectional design which lacks information about temporality.

To the best of our knowledge, this is the first longitudinal study with adolescents to examine the relationship between CPF and self-reported neurological symptoms. In this study, a gradual increase in neurological symptoms, relative to the baseline time interval, was observed among the applicators from during the CPF application period after accounting for the number of days worked during the week, home use of pesticides by the participant, age, education and family monthly income levels. A significant 30 percentage point increase in the neurological symptoms relative to the baseline time interval was observed on time interval 8 (days 63-77 of the study). This is perhaps due to a second short CPF application episode in the same season in field station 1. Self-reported symptoms among applicators remained significantly elevated from the pre-application period until day 217, approximately five months after the cessation of exposure showing evidence that despite discontinuation of CPF application, repeated exposure of this pesticide led to persistence of neurological health effects for several months. Compared with the applicators, the non-applicators showed relatively late reporting of neurological symptoms,

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3 perhaps due to the environmental CPF exposure. It is interesting to note that the non-applicators
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5 still reported approximately 9 percentage point more symptoms relative to baseline at the last
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7 time interval (day 105-217). Residual CPF can survive in indoor environments for an extended
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9 period of time, can rapidly bind to soil and plants and has a half-life of several months in soil ³⁷
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11 ³⁸. We anticipate that because of these properties, CPF remained in the environment as a
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13 potential source of environmental exposure leading to increased symptom reporting among non-
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15 applicators.
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22 The symptom reporting over time showed a recovery phase at time interval 10 (day 105-207)
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24 when percentage of symptom reporting relative to baseline declined substantially from the
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26 previous time intervals (Table 3, Figure 2). Using the same sample, we recently demonstrated
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28 that both the applicators and non-applicators experienced peak median BChE depression during
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30 the CPF application period but BChE returned to the baseline level by the end of the study (day
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32 217/January 5, 2011) ³². We anticipate that symptoms were following BChE activity pattern, i.e.,
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34 as the BChE activity was returning back to the baseline level neurological symptoms were going
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36 through the recovery phase.
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45 Prior to this study, a cross-sectional study on Egyptian cotton field workers reported
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47 associations between OP exposure and neurological symptoms ^{20 21}. Similar to an Indian study on
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49 occupationally exposed adolescents ²², the previous Egyptian adolescent study ^{20 21} presented
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51 descriptive statistics to show the difference between exposed and unexposed adolescents in terms
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53 of the prevalence of various neurological symptoms. However, these studies did not take
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55 potential sociodemographic confounders into account. Results of the present study were
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4 consistent with several longitudinal studies conducted in adult populations. In one study of
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6 occupationally and non-occupationally OP pesticide-exposed farmers and fishermen, delayed
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8 persistence of neurological symptoms were found during the two-year follow-up⁷. Results from a
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10 clinical examination of the same cohort found that there were deficits related to sensory function
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12 ³⁹. Another study, conducted over three years with Colorado farm workers, reported an
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14 association between OP exposure and symptoms of depression ¹². Consistency in the results
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16 across studies indicate that a Q16 based self-reported questionnaire used in all of these studies is
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18 a reliable measure to estimate health effects resulting from OP (in this case CPF) exposure.
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25 Our study is also novel in its approach of including prospective measures of biomarkers.
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27 First, instead of using single-time point biomarker data commonly used in cross-sectional
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29 studies, our study analyzed urinary TCPy levels at multiple time points. The collection of pre,
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31 during and post application samples resulted in a precise estimate of cumulative exposure from
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33 April 11 to January 5 ³². This has enabled us to overcome a historical challenge in characterizing
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35 OP exposure and allows us to subsequently examine the association of cumulative exposure with
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37 neurological symptoms. An additional limitation often encountered by past studies was the
38
39 absence of established baseline AChE and BChE levels. A recent adult study examining the
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41 variation of cholinesterase levels among OP pesticides and carbamate-exposed field-workers
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43 could not establish any baseline AChE/BChE due to the mobility of the migrant study population
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45 ⁴⁰. Another Egyptian adolescent study also reported greater reduction of AChE activity among
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47 the pesticide applicators compared to the controls ^{20 21}. By collecting blood samples prior to the
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49 start of the application season, baseline data were established, which allowed us to compute more
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3 precise measures of change in activities of AChE and BChE from pre-exposure to post-exposure
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10 Two previous studies of Kenyan and Palestinian farm workers, which measured
11 cholinesterase levels before and after exposure, found associations between cholinesterase
12 inhibition and respiratory, eye and neurological symptoms^{9 41}. Potential occupational
13 confounding factors (e.g residential application of pesticides and number of days worked in
14 agriculture) that are associated with neurological symptoms^{20 42} were not taken into account
15 while examining exposure-outcome associations in these past studies. These potential
16 confounding variables were included in our study questionnaires and later examined during
17 statistical analysis.
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31 We identified a comparison group (non-applicators) who were similar in demographic
32 characteristics to our applicators. It is often true that control groups in occupational settings may
33 not be truly unexposed¹. In our study, close proximity to the agricultural field (less than 25m)
34 and application of pesticides at home were the two environmental factors offering potential
35 exposure opportunities to the non-applicators as indicated by elevated urinary TCPy levels
36 during the period of CPF application to cotton fields³². To encounter this potential confounder,
37 all statistical models were adjusted for these two variables in addition to other sociodemographic
38 variables.
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51 It is difficult to explain why we found no relationship between TCPy and neurological
52 symptoms among the non-applicators when a delayed effect of environmental or passive CPF
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3 exposure on symptoms was evident among this subgroup in the corresponding GEE model
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5 (Table 3 & Figure 2). One possible explanation is that the range of cumulative exposure was
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7 much lower among the non-applicators (154 to 24,180 mg/g creatinine; median 2591 mg/g
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9 creatinine) compared to the applicators (232 to 28,260 mg/g creatinine; median 10318 mg/g
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11 creatinine). Small sample size and differences in cumulative exposure might have contributed to
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13 the non-significant association in the non-applicator subgroup. Some other undocumented
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15 environmental factors such as working during high temperatures along with carrying a heavy
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17 backpack during CPF application might have positively confounded the association among the
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19 applicators.
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27 We acknowledge that we relied on self-reported outcome measure. Therefore, there was a
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29 possibility that the frequent completion of the neurological symptoms survey (32 times over 8
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31 months) could itself have had an influence on the increase in symptoms during the CPF
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33 application season. This could partially explain why these symptoms were not associated with
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35 TCPy levels among non-applicators.
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42 The non-specific nature of many of the symptoms is another limitation of the current study,
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44 the biological significance of these self-reported symptoms is unknown. However, the goal of the
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46 study was not to establish that more symptoms lead to development of any neurological disease.
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48 Rather we attempted to examine how repeated or cumulative exposure to CPF determined the
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50 pattern of neurological symptoms over the entire season. Five of the symptoms included in our
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52 questionnaire are considered non-specific, including, headache, dizziness, fatigue, loss of
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54 consciousness and insomnia. The remaining 20 symptoms were classified into more specific
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3 neurological functions such as behavior, autonomic, sensory, cognitive or motor functions. When
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6 we excluded these non-specific symptoms from the summary measure, the estimated betas for
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8 the associations of TCPy, change in AChE and BChE activities with average percentage of 20
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10 neurological symptoms were found to be 3.19 ($p<0.001$), -6.11 ($p=0.60$) and -9.49 ($p=0.05$)
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12 respectively after accounting for potential covariates.
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20 CONCLUSION

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22 Our study reinforces the need for the development and execution of intervention programs
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24 for the residents of agricultural communities, including pesticide applicators, in developing
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26 countries. Future interventions should include hygiene practices, behaviors and use of protective
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28 equipment, in both occupational and residential environments . Our study is the first to
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30 demonstrate that repeated occupational CPF exposure is an important determinant of
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32 neurological symptoms in adolescent applicators and non-applicators over time, with symptoms
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34 peaking during the exposure season and partly recovering in months following exposure. The
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36 study also showed a significant association between cumulative CPF exposure and symptoms,
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38 using cumulative urinary TCPy as a biomarker of exposure. Future studies are needed to assess
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40 the temporal and dose-dependent effects of repeated CPF exposure on neurological symptoms
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42 and neurobehavioral deficits in children, adolescents and adults to identify the most sensitive
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44 populations.
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11

12 13 14 15 **Contributorship Statement**

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17 All authors have made substantial contributions to conception and design, acquisition of data, or
18
19 analysis and interpretation of data in the study; Dr. Khalid Khan has taken the lead to draft the
20
21 manuscript. Whereas other authors have revised the draft critically for important intellectual
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23 content; All of the authors have provided final approval of the version to be published. Dr. Diane
24
25 Rohlman is the Principal Investigator of the study and has supervised each step of the manuscript
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27 development process. She has been listed as the Corresponding author.
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41 responsibility and does not necessarily represent official views of NIEHS.
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45 46 **IRB Approval:**

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48 The study was approved by the OHSU IRB in June 2009 and by the Medical Ethics committee of
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50 the Faculty of Medicine, Menoufia University in July 2009.
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55 56 **Conflict of Interest Statement**

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4 None of the authors has any potential financial, personal or other conflict of interest, which could
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6 inappropriately influence this study.
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9 **Data Sharing Statement**

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11 Additional unpublished data from the study are available to Dr. Diane Rohlman ([diane-](mailto:diane-rohlman@uiowa.edu)
12 rohlman@uiowa.edu), Dr. Ahmed Ismail (aa-ismail@hotmail.com) and Dr. James Olson ([ismail@hotmail.com](mailto:aa-
13 <a href=)) in Excel or SPSS datasets. They can be reached by email.
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20 Abbreviations: CPF (chlorpyrifos); OP (organophosphorus); TCPy (3,5,6-trichloro-2-pyridinol);

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Longitudinal Assessment of Chlorpyrifos Exposure and Self-Reported Neurological

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Symptoms in Adolescent Pesticide Applicators

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Word Count: 4510

Key Words: chlorpyrifos, neurological symptoms, TCPy, cholinesterase, occupational exposure

ABSTRACT

Objectives: Occupational exposure of organophosphorus pesticides (OPs), such as chlorpyrifos (CPF), in adolescents is of particular concern because of the potential vulnerability of the developing neurological system. The objectives of the study were to examine how neurological symptoms reported over the CPF application season vary across time, whether these effects are reversible post application and if there are any associations between CPF biomarkers and neurological symptoms in an adolescent study population.

Methods: Egyptian adolescent CPF applicators (n=57) and non-applicators (n=38) were recruited for a longitudinal study. Self-reported data for 25 neurological symptoms were collected at 32 time points over the 78-month period before, during and after CPF-application. Urine and blood samples were collected for CPF-specific biomarkers: urine trichloro-2-pyridinol (TCPy), and blood cholinesterase.

Results: When we compared reporting of symptoms between applicators and non-applicators at different time intervals over the 8-month study period, we observed both groups reporting the highest numbers of symptoms in the middle of the CPF application season. We observed the

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9 ~~greatest during increased reporting of neurological symptoms among both applicators and non-~~
10 ~~applicators after several weeks of repeated CPF application.~~ Applicators ~~demonstrated~~ reported a
11 greater percentage of neurological symptoms, relative to baseline, than the non-applicators after
12 accounting for potential covariates. ~~Similar models revealed that~~ Only among the applicators,
13 cumulative TCPy was positively and significantly associated with the average percentage of
14 symptoms, ~~but only among the applicators.~~ Significant Associations ~~associations of between~~ the
15 change in butyrylcholinesterase (BChE) from pre to post application season ~~with and~~ several
16 ~~subclasses domains~~ of neurological symptoms were also found ~~significant or marginally~~
17 ~~significant~~ even after adjusting for potential covariates.

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26 *Conclusions:* These observations reinforce the growing concern regarding the neurotoxic health
27 effects of CPF in adolescent applicators in developing countries and the need for developing
28 and implementing intervention programs ~~the importance of exposure prevention during the~~
29 application season ~~through increased use of personal protective equipment.~~

30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 **STRENGTHS AND LIMITATIONS OF THE STUDY:**

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- This is the first longitudinal study ~~showing the~~demonstrating an association between ~~specific organophosphorus pesticide~~CPF exposure and reporting of neurological symptoms in adolescent applicators.
- ~~Symptoms in applicators are compared with symptoms in non-applicator thus showing the effect of environmental CPF exposure in general population.~~
- The study is also novel in its approach to include prospective measures of biomarkers of CPF exposure and effect and to examine their associations with neurological symptoms.
- The non-specific nature of many of the symptoms is a limitation of the current study.
- Small sample size ~~is another limitation study that~~of this study may have influenced the significance levels of exposure-outcome relationships.

~~Results of the study may be generalizable only to agricultural communities with similar~~

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What this paper adds

- ~~It is not fully understood how neurological symptoms vary across time in adolescents exposed to specific organophosphorus pesticide.~~
- Applicators are more likely to report increased symptoms compared to non-applicators.
- Repeated occupational exposure to CPF increases the reporting of acute neurological symptoms during the CPF application season, ~~and~~ the symptoms ~~may~~ persist for months after the cessation of exposure in both applicators and non-applicators.
- Cumulative biomarker of CPF exposure (TCPy) also demonstrates an association with neurological symptoms in applicators.
- Reduction of CPF exposure among the adolescent applicators and non-applicator residents of agricultural communities should be a public health priority since neurological symptoms remained elevated even after the cessation of CPF application.

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INTRODUCTION

The high prevalence use of agricultural use of organophosphorus pesticides (OPs) has been recognized as a major global public health challenge for agriculture-based communities, due to their associations with adverse neurological outcomes. Immediate ~~or~~ and short-term neurological signs and symptoms ranging from less severe (headache, dizziness, nausea etc.) to more severe (muscle weakness, bronchospasm, change in heart rate etc.) ~~were~~ have all been reported after occupational OP exposure to OPs¹. ~~These short-term symptoms were reported as early as 48 hours after acute exposure~~². Although high levels of occupational OP exposure can be associated with symptoms persisting for several years³, repeated, moderate to low exposures can also produce chronic neurological symptoms and deficits in neurobehavioral performance⁴. Converging evidence regarding the associations between OP exposures and neurological symptoms is based on adult occupational studies with adults conducted in a wide range of study settings. ~~These~~ including comparisons between exposed and non-exposed farmworkers in the US⁵, South Africa⁶, Nicaragua^{7,8}, Kenya⁹, Sri Lanka¹⁰ and Egypt¹¹. ~~These studies have used~~

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~~self reported questionnaire data containing non specific neurological symptoms.~~ Additional evidence for the effect of pesticides on somatic and mood symptoms are also found in the literature^{2 12}.

~~Although it is illegal there have reports of involvement of US adolescents in mixing and applying pesticides in some agricultural communities.^{13 14} The developing bodies of children and adolescents may not break down pesticide as effectively as adult and they may receive a larger dose per unit of body weight for a given exposure due to their smaller body size.¹⁵ making them more vulnerable to neurological effects. Animal and human studies have also suggested that paraoxonase PON-1, an organophosphate detoxifying enzyme, is less active in younger populations making them more vulnerable to OP toxicity.^{16 17} Although less commonly studied, OP exposures were also found to be associated with neurological symptoms in children and adolescents. A recent study has found association of environmental CPF exposure with structural changes in developing brain of the children and adolescents.¹⁸ In developing countries, children and adolescents are engaged in risky agricultural work involving activities including OP the application of OPs, and this presents presenting a major public health concern.¹⁹ Even in the US, adolescents can be involved in mixing and applying pesticides.^{15 16} Because of their smaller body size, the biological doses of pesticides (for children and adolescents may be substantially higher than adults.¹⁷ making making them more vulnerable to neurological effects. Animal and human studies have also suggested that paraoxonase (PON 1) — an organophosphate detoxifying enzyme — is less active in younger populations making them more vulnerable to OP toxicity.^{18 19}~~

~~In two epidemiological studies, Egyptian and Indian children and adolescents living in agricultural communities have demonstrated associations between occupational and~~

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~~environmental OP exposure and neurological and neuromuscular problems²⁰⁻²². An Egyptian cross-sectional study found adolescent pesticide applicators reporting more neurological symptoms and neuromuscular problems than controls.²⁰⁻²¹ Association between environmental OP exposure and neurological symptoms was also demonstrated in children living in an Indian agricultural community.²²~~

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Biomarkers have been used to characterize OP exposure in epidemiological and occupational studies. Urinary trichloro-2-pyridinol (TCPy)²³ is a relatively specific CPF metabolite of CPF exposure, which is eliminated in the urine with a half-life of 27 hr hours following exposure.²³ Due to the ease and non-invasiveness of the collection of urine samples, TCPy is widely recognized as a useful biomarker of exposure, particularly in children and adolescents.^{24 25} The classic mode of OP toxicity is manifested by the inhibition of cholinesterase. Both blood acetylcholinesterase (AChE) and butyrylcholinesterase (BChE) are biomarkers of effect, with BChE being more sensitive to inhibition by OP pesticides.²⁶ A small number of adult studies found associations between inhibition of cholinergic activities with self-reported symptoms^{9 10}; however, this relationship has rarely been examined in adolescent studies.

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Understanding the relationship between OP exposure and the change in neurological symptoms ~~aeross-over~~ across-over time (temporal change) is important because application-related exposure follows a seasonal pattern in most areas. ~~Moreover, specific OP exposure is important to track the changes in symptom reporting over time.~~ Two longitudinal studies with agricultural workers

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9 demonstrated that short-term neurological signs and symptoms were associated with initial acute
10 episodes of exposure, which eventually advanced into long-term sequelae.^{7 12} However, these
11 studies did not characterize exposure ~~and did not identify any specific OP that was being~~
12 ~~applied by identifying specific types of OPs that were related to the symptoms.~~

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19 The primary objective of this study was to determine whether occupational exposure to CPF
20 is associated with self-reported neurological symptoms in adolescents. ~~To investigate whether~~
21 ~~occupational exposure to CPF is associated with self-reported neurological symptoms,~~ Through a
22 prospective study, we compared adolescent applicators exposed to CPF with adolescent non-
23 applicators working and residing in agricultural communities in Egypt ~~through a prospective~~
24 ~~study.~~ Typically, CPF is the primary insecticide used by pesticide applicators in Egyptian cotton
25 fields; ~~including adolescent applicators, and offering~~ us a unique exposure environment
26 opportunity with well characterized occupational exposure. The possibility of potential
27 confounding effects of other neurotoxic pesticides was minimal because of limited use of other
28 pesticides in the study area. ~~We attempted to answer the critical questions of how repeated~~
29 ~~exposures to OP determines reporting of neurological symptoms.~~ The goals of the study were to
30 examine how neurological symptoms vary across-over time during the exposure season, if these
31 effects could reverse at the cessation of exposure and whether there are any associations between
32 OP-CPF biomarkers and neurological symptoms in the adolescent study population. ~~A~~
33 ~~questionnaire was administered pre-, mid- and post-CPF application season to examine changes~~
34 ~~in self-reported symptoms across time.~~

50 METHODS

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Study area and population

Two agricultural districts were selected from A prospective study was conducted in Menoufia Governorate, Egypt (Supplementary Figure 1) to conduct a prospective study from April 2010 to January 2011. Two of the nine districts of Menoufia, Al Shohada and Berket El Sabea were chosen randomly to conduct the study (Supplementary Figure 1). In Egypt, adolescents are hired seasonally to apply pesticides to cotton fields and the schedule of pesticide applications to the cotton crop is regulated by the Ministry of Agriculture. The typical workday was from 8am-12pm and from 3pm-7pm, six days per week. In the year of During 2011-2010, approximately 2100 liters of OPs were applied on approximately to 5700 acres of cotton fields (personal communication with the Ministry of Agriculture). Chlorpyrifos is the primary OP applied in the districts of Menoufia governorate to the cotton crop from mid-June to early August. Although there are slight variations in the timing of CPF application between the two districts (Supplementary Figure 2 Figure 1), the application patterns are consistent across field stations these two areas. The typical workday was from 8am-12pm and from 3pm-7pm, six days per week. Because there is no regulation in Egypt for mandatory use of personal protective equipment (PPE), dermal exposure and inhalation were both considered as the potential route of exposure in this population.²⁰ Recently, Fenske et al,²⁷ reported that dermal exposure and subsequent absorption through the skin accounted for 94-96% of the total dose of chlorpyrifos CPF in Egyptian pesticide applicators.

Recruitment and data collection

Fifty-eight male adolescents aged 12-21, that were hired seasonally by the Ministry of Agriculture to spray pesticides in the cotton fields, were recruited from two field stations districts

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9 in the Menoufia governate (~~i.e. Al Shohada and Berket El Sabea, field station 1 and field station~~
10 ~~2, respectively~~). Forty adolescent non-applicators were recruited through convenience sampling
11 (~~i.e.~~ word of mouth, direct communication ~~with~~ utilizing contacts through the staff from the
12 local Ministry of Agriculture) from the same districts as the applicators for the cotton crop.
13 These adolescents never worked in the cotton fields as pesticide applicators. ~~We excluded~~
14 ~~one~~One adolescent was excluded from the final analysis due to his inconsistency in participating
15 in the study activities and two other subjects-participants were excluded for questionable sample
16 integrity, resulting in a final sample size of 95 (57 applicators and 38 non-applicators). Written
17 informed consent was obtained from all participants and their legal guardian (for those under
18 18). All the subjects were monetarily compensated for their time during the questionnaire survey
19 and biological samples (~\$5 per visit). The study was approved by the ~~OHSU-Oregon Health and~~
20 ~~Science University~~ IRB in June 2009, and by the Medical Ethics committee of the Faculty of
21 Medicine, Menoufia University in July 2009.

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24 Data collection, for both applicators and non-applicators, occurred at the primary field
25 station for each district. Pesticides applicators and supervisors meet in the field stations, which
26 also provides storage area for the pesticides and the equipment used for application.

27 Outcome assessment

28 We developed a ~~multiple time point~~, 25-item, short-term neurological symptom
29 questionnaire on the basis of the widely used Q16 questionnaire²⁸ and a modified version of the
30 Q16 used in a previous study ~~with on~~ licensed pesticide applicators²⁹. The 25 symptoms were
31 grouped into six domains: behavioral, autonomic, cognitive, sensory, motor and non-specific
32 temporary disability (Table 1). ~~The questionnaire had~~ ~~There were~~ five ~~frequency choices~~ response

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options (0-4) for each symptom ranging from “never” (coded as 0) to “everyday of the week” (coded as 4). Since more than 90% of the responses were between 0-2 (1=once a week and 2=once in every 2-3 days) we recoded each of the symptom response to “0” or “never” and “1” or “at least once a week or more.” Self-reported neurological symptom counts were collected at 32 irregularly spaced dates over an eight-month period spanning from early June 2010 through early January 2011. Beginning on June 2 of 2010 through January 2011 participants reported symptoms occurring in the past week. These time points spanned through this symptom questionnaire administered 32 separate times, at least once per week) and ranged across three spanning over all relevant application periods in the season (different time periods: pre-application, during application, and post-application). For each time point, The the number of positive responses (a response was considered positive and coded as “1” when the participant reported the frequency of the symptom “at least once a week or more”)-was totaled for each person to yield a score ranging from 0–25; division by 25 produced the proportion of symptoms endorsed at each of the 32 time points. This outcome variable was used to compare the change of symptoms over time between applicators and non-applicators. These sample periods were All these time points were collapsed into 10 separate non-overlapping intervals lasting between one and four weeks in length (Supplementary Figure 2 Figure 1). Symptom data during the pre-application period, including the from the first three dates (i.e first fifteen days of the study from June 2 to June 16), when no CPF was applied, was collectively taken to represent the baseline time interval (or time interval 1), against which symptom Symptom reporting from the other nine remaining time intervals was evaluated against time interval 1. The next 5 time intervals, In five of these nine time intervals (between June 19 and July 21), were during the application period of CPF was reported in both field stations. The remaining XX4 time intervals occurred between

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~~XX~~July 24, 2010 and ~~XX~~January 5, 2011 and reflect the post-application period although a brief CPF application was reported in the district where field station 1 was located, and thisThe proportions of symptoms over all the 32 time points was averagedwere averaged across the 32 collection points to produce a season-level average percentage of neurological symptoms over the entire study periodmean proportion of self-reported symptoms. This outcome variable was used to examine the relationships between the biomarkers (TCPy, AChE and BChE) and symptoms. Participants also completed a questionnaire at during baseline addressing their socio-demographic status, household and occupational use of pesticides, including such as number of days of pesticide application or mixing, medical history, safety practices and lifestyle activities including smoking status, hours of sleep at night, number of drinks containing caffeine.

Table 1. Domains of neurological symptoms

Domains	Symptoms
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Behavioral Symptoms	1. Tense or anxious [#] 2. Excessively angry or irritable ^{*#} 3. Depressed or withdrawn ^{*#}
Autonomic Symptoms	4. Nausea [#] 5. Heavy sweating ^{*#} 6. Loss of appetite [#] 7. Fast heart rate ^{*#} 8. Excessive salivation
Cognitive Symptoms	9. Difficulty concentrating ^{*#} 10. Being absentminded and memory problem ^{*#}
Sensory Symptoms	11. Difficulty seeing at night [#] 12. Blurred or double vision [#] 13. Numbness in hands and feet [#] 14. Sense of smell or taste change [#] 15. Ringing in ears
Motor Symptoms	16. Difficulty with balance [#] 17. Weakness in arms and legs [#] 18. Involuntary movement of arms and legs [#] 19. Shaking in hands [*] 20. Difficulty speaking [#]
Temporary Disability (non-specific symptoms)	21. Dizziness [#] 22. Headache ^{*#} 23. Momentary loss of consciousness [#] 24. Fatigue ^{*#} 25. Insomnia [#]

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*Symptoms used in Q-16²⁷

#Symptoms used in Agricultural Health Study²⁸

Urine collection and analysis

Spot urine samples were collected in new and individually wrapped cups at the beginning of the work shift. Urine was collected in new wide mouth plastic cups at eight time points between April 2010 and January 2011. The cups were opened at the time of sample. We collected spot urine samples at the field station at the beginning of the work shift. The collection. Urine cups samples were subsequently transferred to the laboratory at Menoufia University in a cooler with wet ice. At the laboratory, 4 ml aliquots of urine were transferred into labeled 5 ml cryovials within hours of sampling and stored at -20 °C. The banked urine samples were express mailed on dry ice to the University of Buffalo laboratory for analysis of pesticide metabolites; duplicate samples were retained in the -20 °C freezer at Menoufia University. Urine samples in the field station at Berket El-Sabea district² were collected one day after the collection date of the field station at Al-Shohada¹.

Creatinine concentrations were measured using the Jaffe reaction³⁰. The method of urinary TCPy measurement (a primary metabolite of chlorpyrifos) has been described elsewhere²⁴. Briefly, samples were analyzed using gas chromatography-mass spectrometry (negative-ion chemical ionization) and utilized ¹³C-¹⁵N-3,5,6-TCPy as an internal standard. Samples were hydrolyzed with HCl, extracted with toluene, and derivatized using N-(tert-butyldimethylsilyl)-N-methyltrifluoro-acetamide (Sigma Aldrich, USA). A spiked quality control (QC) sample was routinely run with the analytical samples and the metabolite concentration was determined from a standard curve for the peak area for the selective ion. The QC samples consisted of lab samples that were first analyzed for TCPy and the levels were non-detectable. The TCPy standard curve

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was linear from 1-200 ng/ml with a correlation coefficient of 1.000. Samples spiked with 50ng of TCPy/mL (n=20) gave an average metabolite recovery of 94.8% (range = 92 - 98%; SD = 0.931; RSD% = 1.965). A 1ng TCPy/ml spiked sample was run 10 times and the within series RSD% = 1.6. The minimum detection level was 0.5 ng/ml of urine. Briefly, negative ion chemical ionization gas chromatography-mass spectrometry was used that utilized ^{13}C - ^{15}N -3,5,6-TCPy as an internal standard. Jaffe reaction was used for colorimetric analysis of creatinine.³⁰ The within-run imprecision of this assay is very low (<2% coefficient of variation and an intra-class correlation coefficient of 0.997). The quality control (QC) samples consisted of lab samples that were first analyzed for TCPy levels; these levels were non-detectable. Twenty aliquots were then spiked with 50ng of TCPy/mL of urine; these were then extracted and analyzed as per protocol. The recovery rates ranged from 92%–98% with the average being 94.8%, SD = 0.931 and the CV% = 1.965, minimum detection level was 0.0501 ng. QC replicates had 94.75% recovery. Finally, cumulative urinary TCPy for each participant was determined by calculating the area under the curve for the plotted values for eight time intervals.

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Blood collection and ChE analysis

To establish the baseline ChE activity, pre-application blood draws occurred on April 11 and June 2, 2010, prior to the start of the official government-regulated CPF application season. As with the urine collection, blood draws occurred in the field station at Berket El-Sabea² were performed one day later. Changes in both AChE and BChE levels from baseline to the end of CPF-application season (blood collected on September 4, 2010) were estimated. Blood samples were collected by venipuncture into 10mL lavender top (EDTA) vacutainer tubes and

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9 immediately placed on wet ice and transported to Menoufia University, where they were
10 analyzed in duplicate for AChE and BChE activity using an EQM Test-Mate kit (EQM Research
11 Inc., Cincinnati, OH, USA) as described previously.²⁴

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16 17 **Statistical analysis**

18 We used SPSS version 18.0 and STATA (version 11; Stata Corporation, College Station,
19 TX) for the statistical analysis. Sociodemographic variables were summarized and described
20 using means and standard deviations for continuous responses and percentages for discrete
21 outcomes; simple comparisons between applicators and non-applicators were completed using t-
22 tests (~~continuous measures~~) or chi-square tests (~~discrete outcomes~~). To calculate the value of

23 cumulative TCPy for each participant we used STATA's pharmacokinetic function (pkexamine)
24 to employing the trapezoid rule to estimate the area under the curve for each participant over the
25 study time. By definition, cumulative TCPy was the sum of the concentration at each time point

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26 multiplied by the duration between time points. This variable reflects the total amount of TCPy
27 excreted over the study period for which urine was collected and assayed. Concentrations of

28 cumulative TCPy, AChE and BChE exhibited pronounced right skewed distributions and

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29 more than a 3-fold separation between the minimum and maximum observed values;

30 consequently, these responses were log_e-transformed prior to analysis to improve symmetry.

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31 Both AChE and BChE were expressed as a log-transformed ratio of post-application activity

32 relative to pre-application activity. Then the associations between the change of these

33 cholinesterase markers from pre to post application seasons and self-reported symptoms were

34 examined using linear regression models that took potential covariates into account. Similar

35 regression analyses were used to examine the relationship between cumulative TCPy and

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~~neurological symptoms. prior to investigation of associations with average percentage of self-reported symptoms.~~ All p-values are two-sided with significance judged relative to a 0.05 level.

Spearman correlation coefficients were used to estimate associations between urine and blood biomarkers and symptom scores. Generalized estimating equations (GEE)³¹ were used to model the proportion of neurological symptoms reported in each time interval while controlling for number of days worked (within five days of the symptom reporting date), home use of pesticides, age, education and income levels. The one fitted model was used to estimate changes over time, relative to the first time interval (June 2–June 16), for applicators and non-applicators, as well as to examine whether changes relative to baseline differed between the two groups (via group-by-time interaction).

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RESULTS

Sociodemographic Characteristics

Ninety-two of the participants (97%) were between 12 and 18 years old with the remaining three between 19 and 21. The two groups, non-applicators and applicators, did not differ significantly in terms of age, educational status, family income, number of people in house, years of pesticide use at home, and insecticides and rodenticides use at home (Table 2). Compared to non-applicators, a significantly higher number of applicators lived close to the field (within 25 meters), had carpet in their homes and applied herbicides at home. Applicators had

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significantly lower BMI than non-applicators. On average, applicators had been working in the field for a little over 3 years.

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Table 2: Sociodemographic characteristics for participants at baseline

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Variables	Non-applicators (n=38)	Applicators (n=57)
	Mean (SD)	Mean (SD)
Age	16.6 (2.4)	16.2 (1.6)
Education	9.8 (1.8)	9.9 (1.8)
Height (cm) ^{###} (34 non-applicators vs 30 app) ^E	166.3 (12.0)	163.4 (10.0)
Weight (kg) ^{###} (34 non-applicators vs 30 app)*	62.0 (15.4)	54.2 (8.6)
BMI (kg/m ²) ^{###} (34 non-applicators vs 30 app)*	22.1 (3.7)	20.2 (2.2)
Number of people in house	5.6 (1.1)	6.0 (1.8)
Home pesticide use (years) ^{###} (19 Non-applicators & 44 App)*	1.6 (1.9)	2.5 (1.9)
Occupational application of pesticides (yrs)	-	3.1 (1.5)
Days/week of pesticide application	-	4.8 (1.3)
Hours/day of pesticide application	-	5.2 (0.7)
	% (n)	% (n)
Family Monthly Income (<500 E) ^{###} (Low)	78.9 (30)	71.9 (41)
Applied pesticides in home in last 5 yrs (yes)*	47.4 (18)	78.9 (45)
Computer use (once a week or more)*	65.8 (25)	45.6 (26)
Carpet in house (yes)*	27.0 (10)	54.4 (31)
Live close ^{###} within 25m to agricultural field (yes)*	23.7 (9)	50.9 (29)
Types of pesticides applied at home ^{###} (24 vs 49)		
Herbicides*	13.0 (3)	44.9 (21)
Insecticides	83.3 (20)	93.9 (46)
Rodenticides	16.7 (4)	14.3 (7)

*p<0.05 for group difference; ^{###}30 applicators vs 34 non applicators; ^{###}44 applicators vs 19 non applicators; ^{###}49 applicators vs 24 non applicators. E=Egyptian pound

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Comment [DR1]: How is this defined? What is low income – put as a footnote?

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Change in symptoms over time

We considered days 0-14 as the baseline time interval (1st-time interval 1) when no application of CPF was reported. While examining symptoms reported over time among both applicators and non-applicators we took various potential confounders into account. These include occupational factors such as days worked per week in pesticide applications, number of years of pesticide use at home and socio-demographic factors such as age, education and income level of the participants. Applicators began increased reporting of neurological symptoms at the beginning of the ~~chlorpyrifos-CPF~~ application season (at the 2nd-time interval 2 between days 17-21 of the study). The percentage of neurological symptoms ~~increased~~ continued to increase during the application season and reached the peak at the 6th-time interval 6, representing days 45-48, the time when ~~the chlorpyrifos~~ CPF application period ended. This was followed by a drop of symptom reporting indicating a small recovery due to the cessation of exposure in both districts. The highest peak of symptom reporting was observed at the 8th time interval 8 representing days 63-77 (Table 3). This happened perhaps due to a small episode of CPF application in field station 1 (between time intervals 7 and 8). Similar to the applicators, the non-applicators also demonstrated the highest increase in the proportion of neurological symptoms during the 8th time interval 8 although the magnitude of the change was smaller (14 percentage point increase of symptoms relative to baseline interval). The change of neurological symptoms relative to baseline declined over the next two time intervals (9th and 10th) in both groups indicating a recovery phase during post-application. For applicators, the percentage of

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reported symptoms at each of the nine subsequent time intervals was always higher than the percentage observed at baseline; non-applicators, by contrast, had a pattern of percentage of reported symptoms that both increased and decreased relative to the baseline time interval (pre-application) over the course of the study (Figure 42).

When applicators and non-applicators are compared with respect to change in percentage of symptoms (relative to baseline), it was always the case that the change (percentage point change relative to baseline) for applicators was greater than the corresponding change for non-applicators even after adjusting for the covariates (Table 3).

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Table 3. Estimated change (95% CI) from baseline in the percentage points of neurological symptoms reported at each of nine successive collected time points, shown separately for non-applicators and applicators.

DOES GREY AREA INDICATE WHEN CPF WAS APPLIED?

Time Intervals	Non-Applicators		Applicators		Change in difference between applicators and non-applicators relative to difference in baseline	
	Adjusted Models*		Adjusted Models*			
Days from Baseline (Day 0-14)	b (% of Symptoms)	p-value	b (% of Symptoms)	p-value	b (% of Symptoms)	p-value
June 2-June 14, 2010	(95% CI)		(95% CI)		(95% CI)	
17-21	-2.74 (-4.61,-0.86)	0.004	4.08 (0.18,7.97)	0.040	6.81 (2.47, 11.15)	0.002
Jun 19-Jun 23						
24-28	-2.68 (-5.18,0.17)	0.004	12.57 (7.75,17.38)	<0.001	15.25 (9.80, 20.69)	<0.001
Jun 26-Jun 30						
31-35	4.45 (-0.80,9.71)	0.10	14.06 (9.21,18.90)	<0.001	9.60 (2.44, 16.77)	0.009
Jul 3-Jul 7						
38-42	-2.28 (-4.75,0.17)	0.07	22.83 (19.25,26.40)	<0.001	25.11 (20.79, 29.43)	<0.001
Jul 10-Jul 14						
45-48	0.46 (-2.37,3.30)	0.75	28.80 (24.27,33.35)	<0.001	28.35 (22.98, 33.72)	<0.001

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	Jul 17-Jul 21						
7	52-59	4.95 (1.63,8.28)	0.003	24.01 (19.92,28.09)	<0.001	19.05 (13.80, 24.30)	<0.001
	Jul 24-Jul 31						
8	63-77	14.49 (11.45,17.55)	<0.001	30.10 (26.53,33.66)	<0.001	15.60 (10.95, 20.26)	<0.001
	Aug 4-Aug 18						
9	80-94	12.08 (8.72,15.44)	<0.001	29.17 (25.22,33.13)	<0.001	17.09 (11.93, 22.25)	<0.001
	Aug 21-Sep 4						
10	105-217	9.22 (6.12,12.32)	<0.001	18.45 (14.30,22.59)	<0.001	9.22 (4.12,14.33)	<0.001
	Sep 22-Jan 5 [#]						

*Models adjusted for number of days worked for applying pesticides, years of pesticide use at home, age, education and income level. CPF application time intervals are shaded in grey. *Estimates have been adjusted for number of days worked applying pesticides, home use of pesticides, age, education and income level. CPF application time intervals are shaded in grey.

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Associations of neurological symptoms with biomarkers

TCPy was detected in 100% of the samples. Summary statistics for TCPy, AChE and BChE of the study samples have been already reported by Crane et al. (2013).³² Mean creatinine concentration of the urine samples was reported to be 1696 μ g/ml with a maximum of 4199 and a minimum of 164 μ g/ml. In brief, the applicators had much higher mean and estimated median peak TCPy concentration than the non-applicators (mean: 719 vs 44.9 μ g/g creatinine; estimated median 137 vs 19.7 μ g/g creatinine). In our study sample, BChE was found to be more sensitive to CPF exposure than AChE, with median activity reduced by 37% from baseline in applicators and 13% in non-applicators during the CPF application period.

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A scatter plot of cumulative TCPy (ug/g creatinine) against average percentage points of symptoms revealed distinct exposure-response gradients by pesticide application status (applicators vs non-applicators) (Supplementary Figure 3a2aFigure 3). In addition, two other scatter plots of change in AChE activity and change in BChE activity from pre-application to post-application against percentage of symptoms also revealed effect measure modification by pesticide application status (Supplementary Figures 3b-2ba & 3e2eb). Therefore, separate linear models for applicators and non-applicators were used to examine the associations of these three biomarkers with ~~the outcome measure~~ symptoms.

Log-transformed TCPy was positively associated with the average percentage of neurological symptoms in the regression models after adjusting for other covariates that may confound exposure-outcome relationship such as field stations, age, family monthly income, ~~home~~ pesticide use at home and average number of hours worked in the field among applicators (b=2.68, p=0.007). However, non-applicators demonstrated positive, but statistically non-significant, associations between TCPy and symptoms. Among applicators, AChE and BChE activity was negatively and significantly associated with the average percentage of neurological symptoms in the unadjusted models. In the adjusted models these associations remained negative but became non-significant (Table 4).

Table 4. Summary of regression analysis for biomarkers of exposure & effect of chlorpyrifos predicting average percentage of neurological symptoms over the entire study stratified by applicator status

Explanatory variables	Unadjusted Models			Adjusted Models*		
	B (se)	95% CI	p-value	B (se)	95% CI	p-value

For Non Applicators

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Ln TCPy (mg/g Cr) (n=28)	0.29 (0.76)	-1.26,1.84	0.71	0.57 (0.79)	-1.06,2.20	0.47
Ln (Post AChE/Pre AChE) (n=21)	-1.25 (16.41)	-35.59,33.1	0.94	-6.57 (18.80)	-43.64,33.50	0.73
Ln (Post BChE/Pre BChE) (n=21)	2.23 (7.04)	-12.51,16.98	0.76	2.50 (7.63)	-13.77,18.77	0.75
<i>For Applicators</i>						
Ln TCPy (mg/g Cr) (n=42)	4.56 (0.63)	3.29,5.84	<0.001	2.68 (0.93)	0.78,4.57	0.007
Ln (Post AChE/Pre AChE) (n=28)	-24.21 (12.79)	-50.50,2.09	0.07	-11.60 (12.44)	-37.46,14.25	0.36
Ln (Post BChE/Pre BChE) (n=29)	-14.52 (4.61)	-23.97,-5.07	0.004	-7.33(5.93)	-19.63,4.97	0.23

*Regression models adjusted for field stations, age, family monthly income, home pesticide use at home and average number of hours of work in the field over the entire application season (for applicators only)

When we examined the biomarker-symptom relationship by subclasses-domains of symptoms among the applicators, we observed significant positive associations of log-transformed TCPy with behavioral, autonomic, cognitive, motor and sensory problems after accounting for sociodemographic and occupational covariates (Supplementary Table 15). The magnitudes of associations (adjusted betas) were greater for autonomic, cognitive and sensory symptoms than the two other subclasses-domains. Although the log-transformed change in AChE activity was not associated with any of these subclasses, change in BChE activity demonstrated a significant association with average percentage of behavioral symptoms (p=0.04) and a marginally significant association with average percentage of cognitive symptoms (p=0.07) €

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Supplementary Table 1).

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Table 5. Summary of regression analysis for biomarkers of exposure and effect of chlorpyrifos predicting average percentage of neurological symptoms by subclasses among Applicators

Outcome variable (Symptom Subclass)	Unadjusted Model		Adjusted Model		Unadjusted Model		Adjusted Model		Unadjusted Model		Adjusted Model	
	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value	B (se)	p-value
Behavior & Affect	2.16 (0.57)	<0.001	2.80 (0.91)	0.004	-12.59 (10.86)	0.26	-16.34 (16.04)	0.33	-6.96 (4.09)	0.10	-12.26 (5.73)	0.04
Autonomic	5.69 (0.75)	<0.001	3.24 (0.99)	0.002	-38.45 (14.88)	0.02	-4.84 (17.22)	0.78	-19.65 (5.42)	0.001	-9.69 (6.40)	0.14
Cognitive	6.92 (0.87)	<0.001	5.27 (1.34)	<0.001	-46.22 (17.49)	0.01	1.73 (20.82)	0.94	-24.16 (6.03)	0.001	-13.56 (7.55)	0.07
Motor	2.48 (0.49)	<0.001	1.67 (0.74)	0.03	-6.27 (8.41)	0.46	4.50 (12.03)	0.37	-5.75 (3.15)	0.08	-3.90 (4.64)	0.41
Sensory	6.47 (0.90)	<0.001	4.94 (1.34)	0.001	-27.49 (17.64)	0.13	-0.75 (22.46)	0.97	-20.00 (6.41)	0.004	-13.88 (8.86)	0.13
Temporary Disability (Non-specific)	5.48 (0.61)	<0.001	3.64 (0.78)	<0.001	-41.13 (14.14)	0.007	-10.74 (16.61)	0.53	-21.80 (4.73)	0.001	-13.52 (5.73)	0.03

*Regression models adjusted for field stations, age, family monthly income, pesticide use at home and average number of hours of work in the field over the entire application season (for applicators only)

DISCUSSION

A-The self-reported symptom questionnaire has been globally recognized as the primary method to capture symptom data in exposed populations. The most common questionnaire utilized is the extended or modified versions of Q-16²⁸, which has been used in many international studies including a study with Nicaraguans living close to cotton fields⁸, Sri Lankan farmworkers¹⁰ and Colorado agricultural communities³³. However, time intervals between exposure and collection of symptom data in these studies varied from one month to twelve months^{6 9 10 29 34-36}. Furthermore, the majority of studies have utilized cross-sectional design which lacks information about temporality.

To the best of our knowledge, this is the first longitudinal study ~~on~~ with adolescents to look ~~into~~ examine the relationship between CPF and self-reported neurological symptoms. In this study, a gradual increase in neurological symptoms, relative to the baseline time interval, was observed among the applicators from the 2nd to the 8th time intervals (~~days 24-77 of the study during June 26 to August 18, 2010~~) during the CPF application period after accounting for the number of days worked during the week, home use of pesticides by the participant, age, education and family monthly income levels. A significant 30 percentage point increase in the neurological symptoms relative to the baseline time interval was observed on the 8th time interval (days 63-77 of the study). This is perhaps due to a second short CPF application episode in the same season in the field station 1 at Al-Shohada. Self-reported symptoms among applicators remained significantly elevated from the baseline time interval pre-application period until day 217, approximately five months after the cessation of exposure showing evidence that despite discontinuation of CPF application, repeated exposure of this pesticide led to persistence of

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neurological health effects for several months. Compared with the applicators, the non-applicators showed relatively late reporting of neurological symptoms, perhaps due to the ~~low level~~-environmental ~~chlorpyrifos CPF~~ exposure. It is interesting to note that the non-applicators still reported approximately 9 percentage point more symptoms relative to baseline ~~in at~~ the last time ~~point-interval~~ (day 105-217). Residual CPF can survive in indoor environments for an extended period of time, can rapidly bind to soil and plants and has a half-life of several months in soil.^{37 38} We anticipate that because of these properties, CPF remained in the environment as a potential source of environmental exposure leading to increased symptom reporting among non-applicators.

The symptom reporting ~~aeross over~~ time showed a recovery phase at ~~the 10th~~ time interval 10 (day 105-207) when percentage of symptom reporting relative to baseline declined substantially from the previous time intervals (Table 3, Figure 42). Using the same sample, we recently demonstrated that both ~~the~~ applicators and non-applicators experienced peak median BChE depression during the CPF application period but BChE returned to the baseline level by the end of the study (day 217/January 5, 2011).³² We anticipate that symptoms were following BChE activity pattern, i.e., as the BChE activity was returning back to the baseline level, ~~recovery from the neurological symptoms was taking place. were going through the recovery phase.~~

Prior to this study, a cross-sectional study on Egyptian cotton field workers reported associations between OP exposure and neurological symptoms.^{20 21} Similar to ~~another~~ Indian study on occupationally exposed adolescents,²² the previous Egyptian adolescent study^{20 21} presented descriptive statistics to show the difference between exposed and unexposed

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adolescents in terms of the prevalence of various neurological symptoms. ~~However, these studies did not take potential~~ ~~without taking other~~ sociodemographic confounders into account. Results of the present study were consistent with several longitudinal studies conducted in adult populations. In one study of occupationally and non-occupationally OP pesticide-exposed farmers and fishermen, delayed persistence of neurological symptoms were found during the two-year follow-up.⁷ Results from a clinical examination of the same cohort found that there were deficits related to sensory function.³⁹ Another study, conducted over three years with Colorado farm workers, reported an association between OP exposure and symptoms of depression.¹² Consistency in the results across studies indicate that a Q-16 based self-reported questionnaire used in all of these studies is a reliable measure to estimate health effects resulting from OP (in this case ~~chlorpyrifos~~ ~~CPF~~) exposure.

Our study is also novel in its approach ~~to include~~ ~~of including~~ prospective measures of biomarkers. First, instead of using single-time point biomarker data (~~urinary TCPy~~) commonly used in cross-sectional studies, our study analyzed urinary TCPy levels at multiple time points. The collection of pre, during and post ~~exposure application~~ samples resulted ~~ing~~ in a precise estimate of cumulative exposure from April 11 to January 5.³² This has enabled us to overcome a historical challenge in characterizing OP exposure and allows us to subsequently examine the association of cumulative exposure with neurological symptoms. An additional limitation often encountered by past studies was the absence of established baseline AChE and BChE levels. A recent adult study examining the variation of cholinesterase levels among OP pesticides and carbamate-exposed field-workers could not establish any baseline AChE/BChE due to the mobility of the migrant study population.⁴⁰ Another Egyptian adolescent study also reported

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greater reduction of ~~AChE~~^{acetylcholinesterase} activity among the pesticide applicators compared to the controls.^{20 21} By collecting blood samples prior to the start of the application season, baseline data were established, which allowed us to compute more precise measures of change in activities of AChE and BChE from pre-exposure to post-exposure periods.

Two previous studies of Kenyan and Palestinian farm workers, which measured cholinesterase levels before and after exposure, found associations between cholinesterase inhibition and respiratory, eye and neurological symptoms.^{9 41} Potential occupational confounding factors (e.g residential application of pesticides and number of days worked in agriculture ~~into account~~) that are associated with neurological symptoms^{20 42} were not taken into account while examining exposure-outcome associations in these past studies. These potential confounding variables were included in our study questionnaires and later examined during statistical analysis.

We identified a comparison group (non-applicators) who were similar in demographic characteristics to our applicators. It is often true that control groups in occupational settings may not be truly unexposed.¹ In our study, close proximity to the agricultural field (~~less than 25m~~) and application of pesticides at home were the two environmental factors offering ~~some degree of OP exposure~~^{potential exposure opportunities} to the non-applicators as indicated by elevated urinary TCPy levels during the period of ~~chlorpyrifos-CPF~~ application to cotton fields.³² To encounter this potential confounder, all statistical models were adjusted for these two variables in addition to other sociodemographic variables.

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9 It is difficult to explain why we found no relationship between TCPy and neurological
10 symptoms among the non-applicators when a delayed effect of environmental or passive CPF
11 exposure on symptoms was evident among this subgroup in the corresponding GEE model
12 (Table 3 & Figure 12). One possible explanation is that the range of cumulative exposure was
13 much lower among the non-applicators (154 to 24,180 mg/g creatinine; median 2591 mg/g
14 creatinine) compared to the applicators (232 to 28,260 mg/g creatinine; median 10318 mg/g
15 creatinine). Small sample size and differences in cumulative exposure might have contributed to
16 the non-significant association in the non-applicator subgroup. Some other undocumented
17 environmental factors such as working during high temperatures along with carrying a heavy
18 backpack during CPF application might have positively confounded the association among the
19 applicators.
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32 We acknowledge that we relied on self-reported outcome measure. Therefore, there was a
33 possibility that the frequent completion of the neurological symptoms survey (32 times over 8
34 months) could itself have had an influence on the increase in symptoms during the CPF
35 application season. This could partially explain why these symptoms were not associated with
36 TCPy levels among non-applicators.
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43 The non-specific nature of many of the symptoms is another limitation of the current study.
44 In addition, the biological significance of these self-reported symptoms is unknown. However,
45 the goal of the study was not to establish that more symptoms lead to development of any
46 neurological disease. Rather we attempted to examine how repeated or cumulative exposure to
47 chlorpyrifos CPF determined the pattern of neurological symptoms over the entire season. Five
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of the symptoms included in our questionnaire are considered non-specific, including, headache, dizziness, fatigue, loss of consciousness and insomnia. The remaining 20 symptoms were classified into more specific neurological functions such as behavior, autonomic, sensory, cognitive or motor functions. When we excluded ~~the five~~ these non-specific symptoms from the summary measure, ~~t~~ The estimated betas for the associations of ~~exposure variables cumulative~~ TCPy, change in AChE and BChE activities with average percentage of 20 neurological symptoms were ~~found to be~~ 3.19 (p<0.001), -6.11 (p=0.60) and -9.49 (p=0.05) respectively after accounting for potential covariates.

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~~Our study was conducted in an agricultural community in Egypt, which is relatively where families are primarily middle class to lower middle class. Results of our study may be generalizable only to agricultural communities with similar sociodemographic characteristics.~~

CONCLUSION

~~Our study reinforces the need for the development and execution of intervention programs for the residents of agricultural communities, including pesticide applicators, in developing countries. Future interventions should include address hygiene practices, behaviors and use of protective equipment, in addressing both occupational and environmental residential environments exposures.~~ Our study is the first to demonstrate that repeated occupational CPF exposure is an important determinant of neurological symptoms in adolescent applicators and non-applicators ~~across-over~~ time, with symptoms peaking during the exposure season and partly recovering in months following exposure. The study also showed ~~a~~ significant association between cumulative CPF exposure and symptoms, using cumulative urinary TCPy as a

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9 biomarker of exposure. Future studies are needed to assess the temporal and dose-dependent
10 effects of repeated CPF exposure on neurological symptoms and neurobehavioral deficits in
11 children, adolescents and adults to identify the most sensitive populations. ~~Similar prospective
12 studies with a larger populations are also needed to assess the relationship between these
13 endpoints and biomarkers of exposure, effect and susceptibility, ultimately identifying
14 biomarkers, which may help protect sensitive population.~~

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35 their parents for their participation, Steve Hutton (Dow Agrosciences, Indianapolis, IN) for
36 providing 13C-15N-3,5,6-TCP, Barbara McGarrigle for the urinary TCPy analytical work and
37 the Research Team at Menoufia University.

38 39 40 41 42 43 **Contributorship Statement**

44 All authors have made substantial contributions to conception and design, acquisition of data, or
45 analysis and interpretation of data in the study; Dr. Khalid Khan has taken the lead to draft the
46 manuscript. Whereas other authors have revised the draft critically for important intellectual
47 content; All of the authors have provided final approval of the version to be published. Dr. Diane
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Rohlman is the Principal Investigator of the study and has supervised each step of the manuscript development process. She has been listed as the Corresponding author.

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IRB Approval:

The study was approved by the OHSU IRB in June 2009 and by the Medical Ethics committee of the Faculty of Medicine, Menoufia University in July 2009.

Conflict of Interest Statement

None of the authors has any potential financial, personal or other conflict of interest, which could inappropriately influence this study.

Data Sharing Statement

Additional unpublished data from the study are available to Dr. Diane Rohlman (diane-rohlman@uiowa.edu), Dr. Ahmed Ismail (aa-ismail@hotmail.com) and Dr. James Olson (aa-ismail@hotmail.com) in Excel or SPSS datasets. They can be reached by email.

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Abbreviations: CPF (chlorpyrifos); OP (organophosphorus); TCPy (3,5,6-trichloro-2-pyridinol);

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AChE (acetylcholinesterase); BChE (butyrylcholinesterase); CYP (cytochrome P450)

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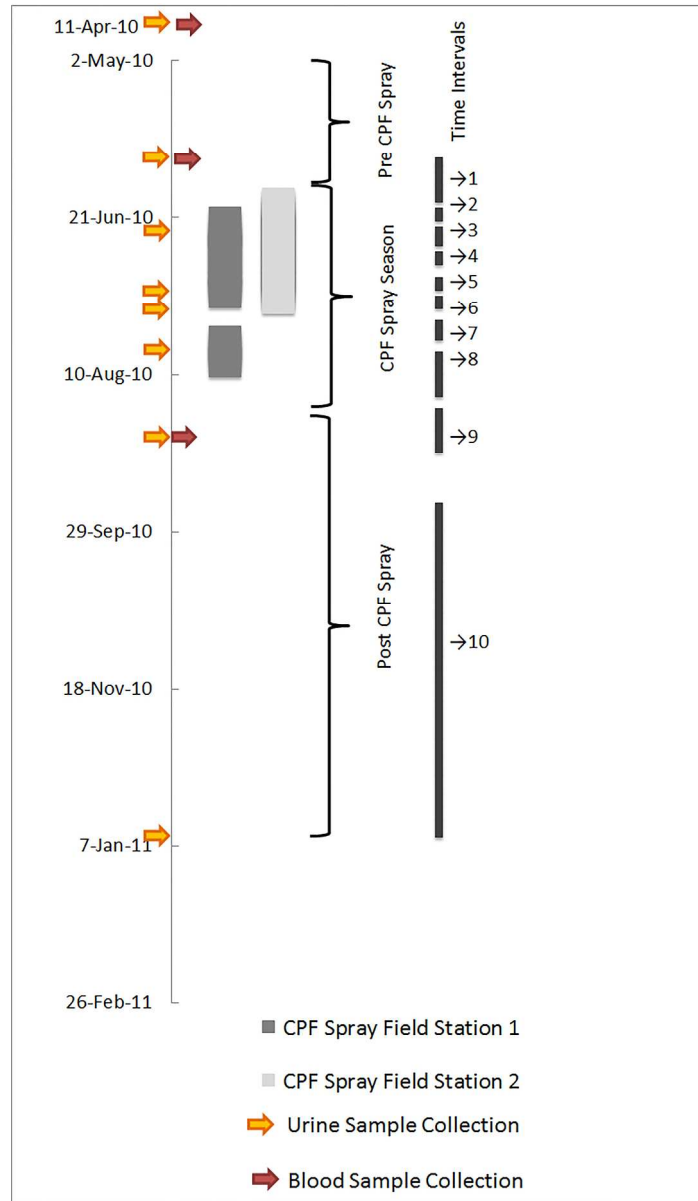


Figure 1. CPF application in the study area showing time intervals in both field stations 1 and 2.

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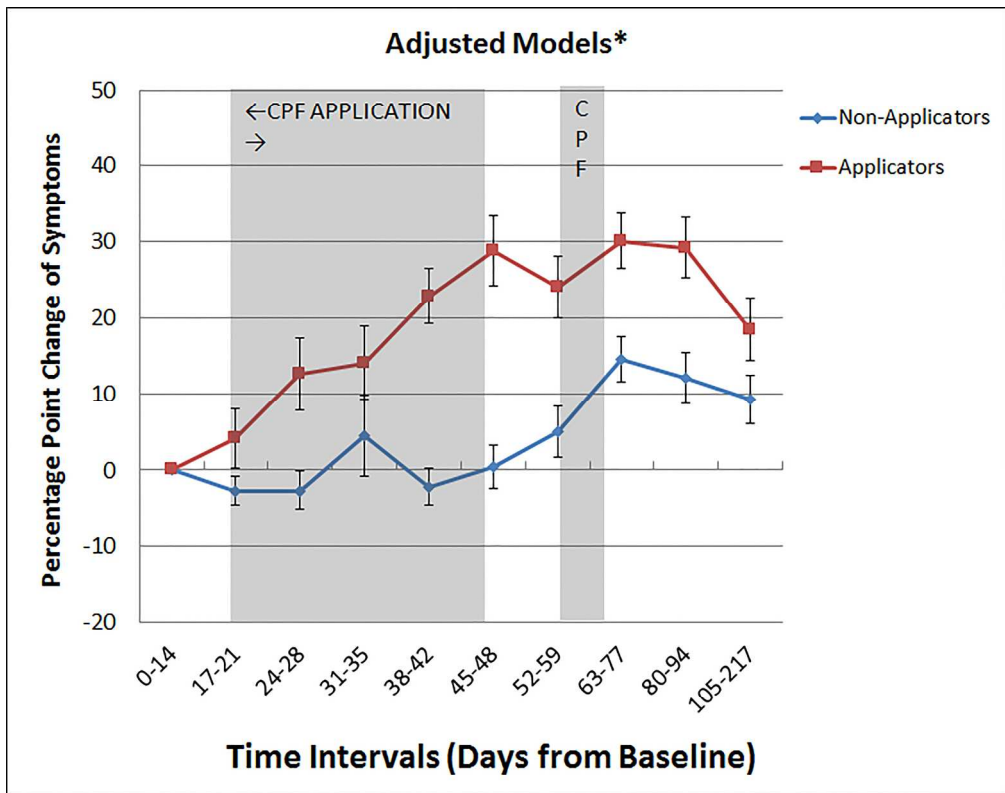


Figure 2. Difference, relative to baseline, in the percentage of symptoms reported at each of nine subsequent time intervals; error bars represent 95% confidence limits for the difference.

Footnote (Figure 2): *Models adjusted for number of days worked for applying pesticides, years of pesticide use at home, age, education and family monthly income. CPF application time intervals are shaded in grey.

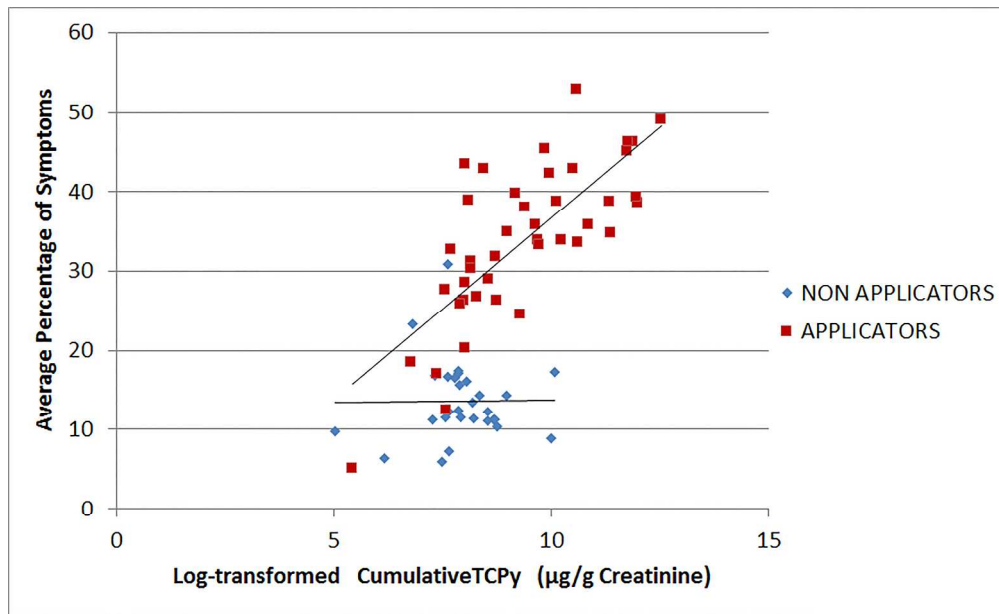


Figure 3. Scatter plots of cumulative TCPy (ug/g creatinine) against percentage of symptoms (n=70)

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STROBE Statement—checklist of items that should be included in reports of observational studies

	Item No	Recommendation
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract: Title indicates "Longitudinal Assessment....." (b) Provide in the abstract an informative and balanced summary of what was done and what was found: Summary provided in the abstract
Introduction		
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported: Explained
Objectives	3	State specific objectives, including any prespecified hypotheses: Objectives specified
Methods		
Study design	4	Present key elements of study design early in the paper: Presented in the last paragraph of the Introduction section
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection: Described in the Method section
Participants	6	(a) <i>Cohort study</i> —Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up: Described in the Method section (b) <i>Cohort study</i> — This is not a matched study; Convenience sampling
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable: Described in Methods
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group: Provided
Bias	9	Describe any efforts to address potential sources of bias
Study size	10	Explain how the study size was arrived at: Explained in Method
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why: Described
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding: Described in statistical analysis section (b) Describe any methods used to examine subgroups and interactions: Described (c) Explain how missing data were addressed: No missing data (d) <i>Cohort study</i> — Not applicable (e) Describe any sensitivity analyses- Not applicable

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Results		
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed: Reported (b) Give reasons for non-participation at each stage: Not applicable (c) Consider use of a flow diagram: Not required as a figure in the result section explained the time-intervals when the participants were observed for the outcome
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders: Provided (b) Indicate number of participants with missing data for each variable of interest: Not applicable (c) <i>Cohort study</i> —Summarise follow-up time (eg, average and total amount): Summarized
Outcome data	15*	<i>Cohort study</i> —Report numbers of outcome events or summary measures over time: Reported
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included: Clear explanation of the estimates are provided (b) Report category boundaries when continuous variables were categorized: Not applicable (c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period: Not relevant
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses: Not performed and therefore not reported
Discussion		
Key results	18	Summarise key results with reference to study objectives: Summary of key findings are presented in the Discussion section
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias: Limitations discussed in the last paragraph of the Discussion section
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence: Provided in the Discussion section
Generalisability	21	Discuss the generalisability (external validity) of the study results: Discussed in the paper
Other information		
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based: Provided after the Discussion section just before the list of references

*Give information separately for cases and controls in case-control studies and, if applicable, for exposed and unexposed groups in cohort and cross-sectional studies: **Not applicable**

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at <http://www.plosmedicine.org/>, Annals of Internal Medicine at <http://www.annals.org/>, and Epidemiology at <http://www.epidem.com/>). Information on the STROBE Initiative is available at www.strobe-statement.org.