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# Development of a questionnaire-based insecticide exposure assessment method and comparison with urinary insecticide biomarkers in young Australian children

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Title: Development of a questionnaire-based insecticide exposure assessment method and comparison with urinary insecticide biomarkers in young Australian children

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Abstract:

Environmental and behavioural factors assessed via an online questionnaire were compared to insecticide metabolite concentrations in urine collected from 61 children from South East Queensland, Australia. Metabolite concentrations (μg/L urine) were transformed using the natural logarithm prior to regression analysis and adjusted for age and creatinine. A significant dietary association was reported for vegetable intake and 3-phenoxybenzoic acid (3-PBA) (β: 1.47 for top quartile of intake versus bottom quartile of intake 95% CI: 0.36, 2.57). Intake of vegetables and fruit were also positively associated with sum non-specific organophosphate metabolites (ΣnsOP). ΣnsOP concentrations were lower when fruits and vegetables were always or almost always washed prior to cooking or eating (β: -0.69 95% CI: -1.25, -0.12). In multivariable modelling 3-PBA concentrations were also associated with hand-washing frequency (β: 1.69 95% CI: 0.76, 2.61 for <1 day versus > 3 day), presence of a dog in the home (β: 0.73 95% CI: 0.07, 1.38), frequency of pest-spray use in the summer months (β: 0.88 95% CI: 0.22, 1.54 weekly versus less than weekly) and season (β: 0.88 95% CI: 0.32, 1.44 for spring/summer versus winter/autumn). This is the first study in Australia to report dietary, behavioural and environmental factors associated with biomarkers of insecticide exposure in young children.

## 1. Introduction

Since the 1940's, synthetic insecticides have been widely used for agricultural and domestic pest-control (Casida and Quistad 1998). Despite major strides being made in the development of insecticide classes that are less persistent in the environment, and more specific to target pests, current use insecticides, including organophosphate (OP) and pyrethroid insecticides, are associated with widespread human exposure and adverse health effects (Abreu-Villaça and Levin 2017). Pyrethroid esters are synthetic chemicals that have structures closely related to the botanical insecticide pyrethrum. There are two groups of synthetic pyrethroids, which are differentiated by the inclusion of a cyano group (type II only). Pyrethroids disrupt the functioning of the nervous system through interactions with voltage-sensitive sodium channels (Soderlund, Clark et al. 2002). They are frequently applied with the chemical synergist, piperonylbutoxide, which prevents the metabolism of pyrethroids by inhibiting cytochrome P450 (CYP 450) monooxygenases, thus prolonging their duration of action. Like pyrethroids, OPs are also neurotoxins, inhibiting the action of the enzyme acetylcholinesterase in the nerve synapses of both insects and mammals, which leads to prolonged and excessive acetycholine signalling (Androutsopoulos, Hernandez et al. 2013). In addition to their intended neurotoxic effects, OPs and pyrethroids disrupt cellular pathways involved in regulation of the cell cycle, cell differentiation, and apoptosis, as well as disrupting normal cellular signalling and metabolic processes (Symonds, Miller et al. 2006, Androutsopoulos, Hernandez et al. 2013).

Young children are at greater risk of both acute (high-dose) and chronic (low-dose) exposure to insecticides than adults. Their different physiological characteristics and behavioural patterns lead to relatively greater exposure. Young children are also more sensitive to toxicant exposure, as their organ systems and detoxification enzymes are immature and still developing (Rice and Barone Jr 2000). The main exposure pathways of young children are shown in Figure 1.

**Figure 1** Major exposure pathways of young children to insecticides: mechanisms for increased exposure risk relative to adults
Increased exposure via inhalation is attributable to higher concentrations of insecticides found in the infant breathing zone, compared to the adult breathing zone, and the relatively greater intake of air by infants (Fenske, Black et al. 1990). Frequent hand-to-mouth behaviour predisposes infants to greater non-dietary insecticide exposure (Melnyk, Byron et al. 2011), and the relatively greater consumption of food by infants compared to adults also contributes to greater dietary intake (Roberts and Karr 2012). Increased contact with contaminants found on the floor in dust, as well as the greater relative surface area of infants, predisposes to greater levels of dermal absorption (Makri, Goveia et al. 2004).

Previous studies have indicated that chronic low-level exposure of Australian children to both OP and pyrethroid insecticides is widespread (Babina, Dollard et al. 2012, Heffernan, English et al. 2016, Li, Wang et al. 2019). Although a growing body of evidence implicates low-level exposure to insecticides during early life associated in a variety of adverse health outcomes, particularly adverse neurodevelopmental outcomes (Bouchard, Chevrier et al. 2011, Koureas, Tsakalof et al. 2012, Rauh, Perera et al. 2012, Roberts and Karr 2012, Raanan, Harley et al. 2014, Shelton, Geraghty et al. 2014), relatively little is known about how young Australian children are exposed to insecticides. More exposure data are needed to characterise the health risk and to identify ways to minimise relevant exposure.

Biomonitoring, the analysis of insecticide metabolite concentrations in urine as a measure of insecticide exposure, has been used with increasing frequency to measure exposure to non-persistent chemicals, including pyrethroid and OP insecticides (Needham, Ozkaynak et al. 2005). Biomonitoring has many advantages, of which the most notable is that aggregate exposure to environmental chemicals may be estimated, even when the sources or pathways of exposure to the parent chemical have not been characterised (Sexton, Needham et al. 2004). However, multiple urine samples are required to accurately classify long-term exposure to chemicals with short half-lives, including insecticides (Sexton and Ryan 2012). Although analytical methods for measuring these chemicals in biological samples are well established, sampling methodology to account for this short-term variation in exposure are not (LaKind, Sobus et al. 2014). In young children, prior to toilet training, special methods for urine collection are required (i.e. paediatric urine bags), which is burdensome to participants, as well as being logistically challenging and resource intensive (Needham and Sexton 2000).

As recently reviewed by our research group, exposure-assessment questionnaires could have several applications, particularly to epidemiological studies in young children where biomonitoring is practically challenging, for the reasons described above (English, Healy et al. 2015). When administered in conjunction with biomonitoring or environmental monitoring, they may also provide important information about potentially modifiable pathways of exposure to environmental toxicants. Although questionnaires have been used extensively to assess pesticide exposure, to our knowledge, there is no questionnaire that has been specifically designed and validated to assess exposure of young children to insecticides (Teitelbaum 2002). The aim of this study was to assess the feasibility of an insecticide-exposure-assessment questionnaire for assessing young Australian children's exposure to insecticides. Since data regarding children's insecticide exposure are scarce in Australia, a secondary aim was to characterise individual levels of exposure of young Australian children to insecticides and examine how exposure may be occurring.

## 2. Methods

### 2.1 Study Design and Sampling

Participants were recruited from the general public, including via posters in public places and email lists, as well as from participants in studies undertaken by our group. The study was conducted from April 2015 to May 2016 in urban areas of Brisbane and Toowoomba, both located in South East Queensland, Australia. Families with children <2 years of age at recruitment were asked to collect two samples during a 48-hour period using paediatric urine collection bags (U-bag® MABIS

Healthcare, Waukegan IL USA), from their enrolled child. Samples were stored in secure biological sample storage packs in participant's home freezers prior to collection by the study team and stored at -20$^{o}$C at the laboratory prior to analysis. The two samples from each child were pooled prior to analysis. Consent was obtained from participant families and ethical approval was obtained from the University of Queensland (2015000397), Australia, and the Children's Health Queensland Human Research Ethics Committee (HREC15QRCH40).

The following insecticide metabolites were included in the analysis:

**Table 1** Insecticide metabolites measured in this study

| Abbreviation | Full name | Parent chemical | Chemical class |
|---|---|---|---|
| DMP | Dimethylphosphate | Various organophosphate insecticides | Organophosphate |
| DMTP | Dimethylthiophosphate | Various organophosphate insecticides | Organophosphate |
| DMDTP | Dimethyldithiophosphate | Various organophosphate insecticides | Organophosphate |
| DEP | Diethylphosphate | Various organophosphate insecticides | Organophosphate |
| DETP | Diethylthiophosphate | Various organophosphate insecticides | Organophosphate |
| DEDTP | Diethyldithiophosphate | Various organophosphate insecticides | Organophosphate |
| TCPY | 3,5,6- Trichloro-2-pyridinol | Chlorpyrifos ; chlorpyrifos-methyl | Organophosphate |
| MDA | Malathion dicarboxylic acid | Malathion | Organophosphate |
| IMPY | 2- Isopropyl-4-methyl-6-hydroxypyrimidine | Diazinon | Organophosphate |
| PNP | *Para*-nitrophenol | Parathion; parathion-methyl | Organophosphate |
| 4F3-PBA | 4-Fluoro-3-phenoxybenzoic acid | Cyfluthrin | Pyrethroid |
| *Cis*-DBCA | *Cis*-3-(2,2-dibromovinyl)-2,2-dimethylcyclopropane carboxylic acid | Deltamethrin | Pyrethroid |
| 3-PBA | 3- Phenoxybenzoic acid | Cyhalothrin; cypermethrin; deltamethrin; fenpropathrin; permethrin; tralomethrin | Pyrethroid |
| *Trans*-DCCA | *Trans*-3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropane carboxylic acid | Permethrin; cypermethrin; cyfluthrin | Pyrethroid |

**2.2 Urinary metabolites**

The methods used in this study were modified from Angerer and Hartwig (2010) and Olsson et al. (2004). The methods in full detail, including quality control methods, are described elsewhere (Li, Wang et al. 2019) . Briefly, for the six DAP metabolites of OP insecticides, 2 mL of samples were spiked with 5 ng of isotopically labelled standards. The samples were then extracted with anhydrous acetonitrile (ACN) and diethyl ether after being freeze-dried overnight. Subsequently, potassium carbonate and pentafluorobenzyl bromide (PFBBr) solution were added into the samples before they were derivatised overnight at 40°C. MilliQ water and *n*-hexane were then added to the derivatised samples before they were mixed on a shaker and centrifuged. The samples were then evaporated under a gentle nitrogen stream to near dryness. After being spiked with the instrument/recovery standard, the samples were analysed using a TRACE GC Ultra coupled to a TSQ Quantum XLS triple quadrupole mass spectrometer equipped with a TriPlus Autosampler (Thermo Fisher Scientific)..

For the other metabolites, 2 mL of each sample was spiked with 5 ng of isotopically labelled standards. To hydrolyse glucuronide or sulphate conjugated metabolites, 1.6 mL of β-glucuronidase (HP-2; purchased from Sigma-Aldrich®) solution was added to the samples to give an activity of ~800 units. The samples were then mixed and incubated at 37°C overnight. The extraction process was accomplished via solid phase extraction (SPE) using hydrophilic-lipophilic balance (HLB) cartridges. After elution and filtration, the filtrates were evaporated to near dryness and spiked with the instrument/recovery standard. Target compounds were analysed using a liquid chromatography (Shimadzu, Nexera 2 UHPLC system, Kyoto, Japan) coupled with a tandem mass spectrometer equipped with an IonDrive source (SCIEX QTRAP® 6500+, Ontario, Canada).

The limit of detection (LOD) for each analyte was calculated as the average plus three times the standard deviation of the levels in blank samples. If a compound was not detected in the blank samples, 3.3 times the instrument detection limit (IDL) was used as the LOD. The LOD for DAPs ranged from 0.0032 to 0.31 ng/mL in urine and for other compounds ranged from 0.00085 to 1.3 ng/mL in urine.

**2.3 Creatinine**

Urinary creatinine was analysed using a liquid chromatography coupled with a tandem mass spectrometer as described elsewhere (He, English et al. 2018).

**2.5 Questionnaire**

The design and pre-testing of the online questionnaire has been previously reported (English, Chen et al. 2017). The questionnaire was pretested with a separate sample of families (n = 5) prior to administration, to minimise error in question interpretation or response. The online exposure tool was self-administered by respondents using Qualtrics (Qualtrics, Provo, UT). The tool included questions pertaining to child-related domains of behaviour, maternal behaviour, consumer attitudes, diet, characteristics of the home, cleaning practices and pets in the home. Using complex skip-logic design, participants only answered questions that were relevant to their children, depending on their home environment and developmental stage. For mouthing behaviours of the child, the respondent was asked two questions, the first was "does your baby mouth (suck or chew on) a variety of objects (including hands) or just a few?" with responses "1. My baby mouths a wide variety of objects 2. My baby doesn't really mouth objects 3. My baby mouths just a few objects." The second was "does your baby like to suck their thumb or fingers?" and the responses were "1. My baby constantly or frequently sucks their thumb or fingers across any given day 2. My baby will usually suck their thumb or fingers at some point during the day, but not constantly 3. My baby only occasionally or rarely sucks their thumb or fingers, but not on a daily basis 4. My baby does not currently show any interest in sucking their thumb or fingers." In addition, respondents were asked to describe their child's consumption of organic foods ("How frequently does your child eat organic food? Organic food is often labelled as "pesticide free' or "certified organic"). Parents were then asked pest-control related domains of questions. To minimise difficulty recalling previous pest-control product use participants were provided with visual aids to recall pests (ants, cockroaches etc.) that may have been treated. Furthermore, questions about specific pest products were associated with pictures representative of the product type, to minimise misinterpretation. Due to the large number of questions included in the questionnaire, questions with poor response rates and or poor distribution of responses were eliminated or condensed, as previously described (English, Chen et al. 2017).

**2.6 Statistical Analysis**

Summary statistics are presented as median and mean and are presented unadjusted (µg/L) and adjusted for creatinine (ng/g). The data distribution was assessed using the skew test and histogram plots. Data were transformed using $\log_e$ to better approximate a normal distribution. For analysis of insecticide concentrations, measures below the limit of detection (LOD) were replaced with the value of ½ LOD. Pearson's correlation coefficient (with $\log_e$ adjusted concentrations) was used to assess correlations between metabolites from the same and different classes. We assessed whether metabolite concentrations were associated in a linear or quadratic fashion with age using a regression model with the following formula:

$$Log\ Concentration = A + \beta_1 * Age + \beta_2 * (Age - Mean\ Age)^2 + \beta_3 * \text{creatinine}$$

Associations between biomonitoring and questionnaire data were assessed using linear regression. The analysis was restricted to specific metabolites with detection frequencies greater than 70% and the sum of the non-specific organophosphate metabolites (ΣnsOP) including DMP, DMTP, DMDTP, DEP, DETP and DEDTP. Age in months and urinary creatinine were included as covariates, as per the recommendation of Barr et al (2005). Further multivariable models were only constructed for 3-PBA, since pyrethroids account for the majority of household insecticide spray products in Australia and are also used for agricultural applications. All data analysis was conducted using Stata statistical software v12.0 (StataCorp, College Station, TX, USA).

## 3. Results

### 3.1 Recruitment

A total of 61 parent-child pairs were recruited from suburban Brisbane (n=59) and suburban Toowoomba (n=2). Sufficient sample volumes for analysis and complete questionnaires were obtained from 56 of the participants, including 28 boys and 26 girls. Of the included children, at the time of completion of the sampling and questionnaire 23 were under the age of 10 months, 20 were aged 10-18 months and 13 were aged 19 months to 26 months. Only 6 children were exclusively breastfed. 85.7% of the participants were consuming solid food regularly. There was no difference in age or sex of excluded versus included participants (mean age included 12.9 months, excluded 14.0 months). Sociodemographic data were not collected.

Metabolites with detection frequencies >70% were PNP (92.9%), TCPy (89.3%), DMTP (76.8%), DCCA (76.8%), 3-PBA (76.8%) and DMP (75.0%), see Table 2. The highest median concentrations were recorded for TCPy (4.86 ug/L), followed by DMP (2.32 ug/L), PNP (2.07 ug/L) and DMTP (1.20 ug/L). The median concentrations of the pyrethroid metabolites 3-PBA and DCCA were 0.46 and 0.35 ug/L, respectively. Creatinine standardised results are shown in Table S1.

**Table 2** Summary results of insecticide metabolite concentrations in urine (ug/L)

| | %>LOD | Mean | Min | P5 | P25 | P50 | P75 | P95 | Max |
|---|---|---|---|---|---|---|---|---|---|
| **DMP** | 75.0% | 7.03 | 0.16 | 0.16 | 0.76 | 2.32 | 7.80 | 37.00 | 50.00 |
| **DMTP** | 76.8% | 4.73 | 0.06 | 0.06 | 0.11 | 1.20 | 3.10 | 33.00 | 56.00 |
| **DMDTP** | 14.3% | 0.77 | 0.48 | 0.48 | 0.48 | 0.48 | 0.48 | 3.70 | 4.87 |
| **DEP** | 37.5% | 2.23 | 0.75 | 0.75 | 0.75 | 0.75 | 2.72 | 8.60 | 11.22 |
| **DETP** | 28.6% | 1.41 | 0.50 | 0.50 | 0.50 | 0.50 | 1.00 | 7.05 | 11.00 |
| **DEDTP** | 7.14% | 0.30 | 0.29 | 0.29 | 0.29 | 0.29 | 0.29 | 0.55 | 0.55 |
| **ΣnsOP** | - | 16.10 | 1.48 | 2.23 | 3.27 | 7.06 | 15.86 | 65.54 | 84.89 |
| **TCPy** | 89.3% | 9.86 | 0.03 | 0.03 | 0.57 | 4.86 | 13.64 | 43.36 | 48.95 |
| **IMPY** | 19.6% | 1.11 | 0.11 | 0.11 | 0.11 | 0.11 | 0.11 | 7.43 | 15.80 |
| **MDA** | 14.3% | 0.06 | 0.03 | 0.03 | 0.03 | 0.03 | 0.03 | 0.26 | 0.65 |
| **PNP** | 92.9% | 2.50 | 0.15 | 0.15 | 1.22 | 2.07 | 3.34 | 6.30 | 13.67 |
| **3-PBA** | 76.8% | 1.30 | 0.04 | 0.04 | 0.10 | 0.46 | 0.93 | 6.27 | 15.20 |
| **F3PBA** | 7.1% | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 | 0.01 |
| **DBCA** | 7.1% | 0.70 | 0.65 | 0.65 | 0.65 | 0.65 | 0.65 | 1.30 | 1.30 |
| **DCCA** | 76.8% | 1.50 | 0.04 | 0.04 | 0.07 | 0.35 | 0.87 | 10.01 | 17.85 |

**LOD: limit of detection. P5-p95: percentile. ΣnsOP: sum of DMP, DMTP, DMDTP, DEP, DETP, DEDTP.**

The pyrethroid and OP metabolite concentrations showed substantial levels of correlation with metabolites from the same class (see Figure S1 and Table S2). For example, TCPy was linearly correlated with DMP (ρ: 0.66, p <0.001), DMTP (ρ: 0.66, p<0.001), and PNP (ρ: 0.38, p = 0.004). 3-PBA and DCCA were also highly correlated (ρ: 0.90, p<0.001). OP and pyrethroid metabolites were also correlated, however, the association was weaker than between metabolites of the same class.

Age (in months) was significantly associated with concentrations of DMP (β:0.10 95% 95% CI: 0.03, 0.17) and DMTP (β: 0.10 95% CI: 0.03, 0.17) and ΣnsOP (β:0.06 95% CI: 0.02, 0.10) (Figure S2). Age had a quadratic association with TCPy concentrations, with peak concentration occurring at approximately 20 months of age.

Linear regression analysis was performed to assess the association between questionnaire data and metabolite concentrations in urine, adjusted for age and creatinine. Children who were walking regularly had lower concentrations of DCCA in their urine (β: -1.98 95% CI: -3.41, -0.56). Mouthing behaviours were examined via two variables. In the first, measuring what objects children mouthed, children who mouthed 'just a few objects' had lower concentrations of TCPy in their urine that children who reportedly 'mouthed a wide variety of objects' (β: -1.327 95% CI:-2.405, -0.249). In contrast, for the second mouthing variable, which asked specifically about frequency of mouthing hands and thumbs, children who exhibited less frequent hand-to-mouth behaviour had higher concentrations of TCPy in their urine. Concentrations of 3-PBA were higher when less-frequent hand-washing was reported (β: 1.63 95% CI: 0.49, 2.77 for hand washing <1/day versus >3/day).

**Table 3** Questionnaire variables and their association with $\log_e$ transformed insecticide metabolite concentrations using linear regression; adjusted for age and creatinine. P-values are given for the variable of interest (not the whole model)

| | N | lnTCPy | lnΣnsOP | lnPNP | lnDCCA | lnPBA |
|---|---|---|---|---|---|---|
| **Walking regularly** | | β (95% CI) | β (95% CI) | β (95% CI) | β (95% CI) | β (95% CI) |
| No: reference | 29 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| Yes | 26 | -0.41 [-1.92, 1.09] | -0.13 [-1.05, 0.80] | 0.46 [-0.39, 1.30] | **-1.98 [-3.41, -0.56]** | -1.32 [-2.64, 0.01] |
| P-value | | 0.99 | 0.08 | 0.28 | **0.01** | 0.05 |
| R2 | | 0.61 | 0.37 | 0.23 | **0.38** | 0.40 |
| **Mouthing behaviour** | | | | | | |
| Mouths a wide variety of objects: reference | 37 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| Doesn't really mouth objects | 8 | **0.82 [-0.47, 2.10]** | -0.57 [-1.34, 0.21] | 0.21 [-0.56, 0.98] | 0.896 [-0.47, 2.26] | 0.84 [-0.37, 2.04] |
| Mouths just a few objects | 11 | **-0.92 [-1.93, 0.10]** | -0.30 [-0.92, 0.32] | 0.02 [-0.57, 0.61] | -0.501 [-1.55, 0.54] | -0.55 [-1.47, 0.37] |
| P-value | | **0.03** | 0.27 | 0.86 | 0.22 | 0.15 |
| R2 | | **0.64** | 0.403 | 0.23 | 0.33 | 0.41 |
| **Thumb/finger sucking** | | | | | | |
| Constant or very frequent: reference | 7 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| Regular | 21 | **0.77 [-0.43, 1.96]** | 0.22 [-0.58, 1.01] | -0.53 [-1.28, 0.21] | 0.15 [-1.24, 1.54] | 0.54 [-0.69, 1.76] |
| Occasional or rarely | 14 | **1.63 [0.35, 2.91]** | 0.63 [-0.20, 1.46] | -0.62 [-1.39, 0.16] | 0.06 [-1.39, 1.51] | 0.50 [-0.77, 1.78] |
| No interest at all | 14 | **2.24 [0.85, 3.63]** | 0.41 [-0.51, 1.33] | -0.43 [-1.29, 0.43] | 0.38 [-1.23, 1.99] | 0.83 [-0.59, 2.25] |
| P-value | | **0.01** | 0.24 | 0.26 | 0.97 | 0.66 |
| R2 | | **0.69** | 0.407 | 0.27 | 0.29 | 0.38 |
| **Hand-washing with soap and water** | | | | | | |
| > 3 /day: reference | 10 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| 1-2 / day | 23 | 0.43 [-0.83, 1.69] | -0.56 [-1.33, 0.21] | 0.20 [-0.54, 0.94] | 1.36 [0.10, 2.63] | **1.41 [0.34, 2.47]** |
| <1 / day | 19 | 0.40 [-0.94, 1.74] | -0.29 [-1.12, 0.53] | 0.04 [-0.75, 0.83] | 1.39 [0.03, 2.74] | **1.63 [0.49, 2.77]** |
| P-value | | 0.67 | 0.31 | 0.78 | 0.09 | **0.02** |
| R2 | | 0.64 | 0.410 | 0.25 | 0.41 | **0.53** |
| **Organic food consumption frequency** | | | | | | |
| Sometimes: reference | 32 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| Rarely or never | 15 | -0.46 [-1.45, 0.54] | -0.19 [-0.84, 0.46] | -0.17 [-0.72, 0.38] | 0.24 [-0.81, 1.28] | -0.04 [-0.97, 0.88] |
| P-value | | 0.21 | 0.55 | 0.53 | 0.65 | 0.93 |
| r2 | | 0.40 | 0.280 | 0.27 | 0.23 | 0.31 |
| **Consumption of bread** | | | | | | |
| Less than weekly: reference | 8 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| About once/week | 8 | 0.66 [-0.95, 2.27] | 0.42 [-0.55, 1.39] | 0.34 [-0.47, 1.14] | -0.57 [-2.18, 1.04] | -0.55 [-1.94, 0.83] |
| About three/week | 14 | 0.93 [-0.59, 2.45] | 0.79 [-0.10, 1.69] | -0.59 [-1.33, 0.15] | -0.87 [-2.35, 0.62] | -1.21 [-2.48, 0.06] |
| About 7/week or more | 17 | 0.79 [-0.93, 2.50] | 1.08 [0.07, 2.09] | -0.26 [-1.11, 0.58] | -1.32 [-3.02, 0.38] | -1.35 [-2.81, 0.10] |

| | N | lnTCPy | lnΣnsOP | lnPNP | lnDCCA | lnPBA |
|---|---|---|---|---|---|---|
| P-value | | 0.20 | 0.4 | 0.40 | 0.48 | 0.42 |
| R2 | | 0.41 | 0.360 | 0.38 | 0.28 | 0.39 |
| **Frequency that fruits and vegetables are washed prior to cooking or eating** | | | | | | |
| Sometimes or never: reference | 22 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| Always or almost always | 25 | 0.51 [-0.41, 1.42] | **-0.69 [-1.25, -0.12]** | -0.05 [-0.56, 0.47] | 0.43 [-0.54, 1.40] | 0.60 [-0.24, 1.44] |
| P-value | | 0.19 | **0.02** | 0.86 | 0.38 | 0.16 |
| R2 | | 0.40 | **0.367** | 0.26 | 0.24 | 0.35 |
| **Consumption of vegetables (lettuce, carrots, tomato, potatoes, corn, pumpkin, broccoli, sweet potato)** | | | | | | |
| Bottom quartile: reference ~4 serves of vegetables/week | 16 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| 2nd quartile ~13 serves of vegetables/week | 15 | 1.02 [-0.51, 2.54] | 0.60 [-0.11, 1.31] | -0.39 [-1.03, 0.25] | -0.04 [-1.18, 1.11] | **0.08 [-0.91, 1.08]** |
| 3rd quartile ~16 serves of vegetables/week | 13 | 0.71 [-0.77, 2.18] | 0.43 [-0.30, 1.17] | -0.46 [-1.12, 0.20] | 0.19 [-1.00, 1.37] | **0.18 [-0.85, 1.21]** |
| 4th quartile ~21 serves of vegetables/week | 12 | 1.27 [-0.17, 2.70] | 1.01 [0.26, 1.75] | 0.15 [-0.56, 0.86] | 1.39 [0.12, 2.66] | **1.47 [0.36, 2.57]** |
| P-value | | 0.28 | 0.06 | 0.85 | 0.066 | **0.04** |
| R2 | | 0.62 | 0.458 | 0.29 | 0.37 | **0.47** |
| **Consumption of fruit (bananas, berries, apples, pears, stone fruit)** | | | | | | |
| Bottom quartile: reference ~1 serve of fruit/week | 16 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| 2nd quartile ~8 serves of fruit/week | 15 | 0.96 [-0.53, 2.46] | **0.02 [-0.69, 0.73]** | -0.01 [-0.66, 0.65] | 0.59 [-0.58, 1.76] | 0.73 [-0.31, 1.76] |
| 3rd quartile ~11 serves of fruit/week | 13 | 0.54 [-1.10, 2.18] | **0.95 [0.15, 1.75]** | -0.19 [-0.91, 0.53] | 0.63 [-0.65, 1.91] | 0.36 [-0.78, 1.49] |
| 4th quartile ~18 serves of fruit/week | 12 | 1.15 [-0.38, 2.68] | **0.53 [-0.25, 1.32]** | 0.06 [-0.66, 0.79] | 1.06 [-0.24, 2.35] | 0.98 [-0.16, 2.13] |
| P-value | | 0.46 | **0.03** | 0.81 | 0.23 | 0.44 |
| R2 | | 0.62 | **0.480** | 0.23 | 0.32 | 0.41 |
| **Frequency of use of pest-control sprays during the summer months** | | | | | | |
| Less than once a week: reference | 43 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| Once a week or more | 13 | **1.05 [0.16, 1.94]** | 0.10 [-0.47, 0.68] | 0.50 [-0.02, 1.01] | 0.77 [-0.18, 1.72] | **0.91 [0.09, 1.74]** |
| P-value | | **0.03** | 0.73 | 0.06 | 0.12 | **0.03** |
| R2 | | **0.63** | 0.371 | 0.28 | 0.32 | **0.42** |
| **Pet dog** | | | | | | |
| No dog: reference | 41 | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) | 0.00 (ref.) |
| One or more dogs | 15 | -0.13 [-1.03, 0.78] | 0.12 [-0.42, 0.67] | -0.01 [-0.52, 0.50] | **1.16 [0.29, 2.04]** | **0.96 [0.16, 1.73]** |
| P-value | | 0.60 | 0.65 | 0.98 | **0.01** | **0.02** |
| R2 | | 0.59 | 0.372 | 0.22 | **0.38** | **0.43** |

Dietary factors assessed due to their potential to modify dietary intake of insecticides included individual food items, as well as consumption of organic food, store-bought food and washing of fruits and vegetables prior to cooking or eating. Following a very limited number of participants reporting consuming exclusively organic food diets, this response was not examined individually, and participants were categorised as those who ate organic food 'sometimes or more frequently', versus 'rarely or never'. There were no significant associations between organic food consumption and insecticide metabolite concentrations. Higher frequency of washing fruits and vegetables was associated with lower ΣnsOP concentrations (β: -0.69 95% CI: -1.25, -0.12). Dietary variables were examined by quartile of consumption. Greater consumption of vegetables (sum of the total intake of lettuce, carrots, tomato, potatoes, corn, pumpkin, broccoli, sweet potato) was associated with higher concentrations of ΣnsOP (β: 1.01 95% CI: 0.26, 1.75 top quartile of intake versus bottom quartile) and 3-PBA (β: 1.47 95% CI: 0.36 to 2.57) in children's urine. Higher consumption of fruit (sum of the total intake of bananas, berries, apples, pears, stone fruit) was associated with higher concentrations of ΣnsOP in children's urine, but the association was not clear as the strongest association occurred in the third quartile of intake.

Pest-control practices in the home were also examined. Increased frequency (once a week or more versus less than once a week) of use of pest-control spray products was significantly associated with both the chlorpyrifos metabolite TCPy concentration (β: 1.05 95% CI: 0.16, 1.94) and the generic pyrethroid metabolite 3-PBA concentration (β: 0.91 95% CI: 0.09, 1.74). Other pest-related questions, including pest-product use patterns, use of a professional pest-controller, attitude towards pests in the home, pest phobias, and whether respondents perceived that pests were a problem in the home were not significantly associated with any of the metabolite concentrations. Presence of a dog in the home was associated with increased concentration of DCCA and 3-PBA in urine (DCCA β: 1.16 95% CI: 0.29, 2.04, 3-PBA β: 0.96 95% CI: 0.16, 1.73). We assessed several variables associated with housing characteristics and quality. Increasing age of the home was positively associated with concentrations of TCPy in urine and renting the home was negatively associated with ΣnsOP, but the associations were not significant. There was no association between flooring types in the home, cleaning practices and biomonitoring results. No indicators of the quality of the home, including peeling paint, water damage, etc. were associated with insecticide metabolite concentrations. Season was only associated with 3-PBA concentrations and is reported in more detail below.

Additional multivariable modelling was conducted only for 3-PBA, a generic pyrethroid metabolite, to account for determinants of exposure: season and organic food consumption. We assessed whether 3-PBA was associated with metabolite concentrations in urine after adjusting for these potentially confounding variables. The base model included the variables previously identified to be significantly associated with 3-PBA concentrations, including a dog in the home, frequency of pest-product spraying, vegetable consumption and hand-washing with soap and water. The base model explained 71% of the total variability in 3-PBA concentrations. Only season was observed to have a significant association with 3-PBA concentrations in the multivariable model, with significantly higher concentrations of 3-PBA being recorded when sampling occurred during spring or summer compared to winter or autumn (β: 0.88 95% CI: 0.32, 1.44). Once season was added to the model, the total variability explained was 77%.

**4. Discussion**

In this study we report associations between environmental, behavioural and dietary factors associated with insecticide metabolite concentrations in urine from young Australian children. Organophosphate concentrations, but not pyrethroid metabolite concentrations, were reported to be positively associated with age.. DMP and DMTP were linearly positively associated with age in months, but TCPy appeared to peak at around 20 months of age. These findings suggest that, with the exception of chlorpyrifos, peak childhood insecticide exposure to organophosphates may not have been captured by the age range included in the study (<2 years at recruitment).

**4.1 Non-specific organophosphate metabolites**

Exposure determinants varied between the insecticide metabolites. For the non-specific OP metabolites consumption of fruits and vegetables were positively associated with urinary concentrations. Elsewhere, consumption of fruits or vegetables has been associated with OP and pyrethroid metabolite concentrations in urine from adults and children in several countries, including the US (Riederer, Bartell et al. 2008, Bradman, Castorina et al. 2011, Morgan and Jones 2013, Chiu, Williams et al. 2018), Germany (Becker, Seiwert et al. 2006), Chile (Munoz-Quezada, Iglesias et al. 2012), France (Glorennec, Serrano et al. 2017) and Spain (Roca, Miralles-Marco et al. 2014). The positive association between concentrations of non-specific OP metabolites with age may be explained by increasing dietary solid food intake that occurs following weaning. In addition, increased frequency of washing of fruits and vegetables prior to cooking or eating was associated with lower non-specific OP metabolite concentrations. Experimental studies have demonstrated that washing fruits and vegetables in tap water is associated with a significant reduction of 30-40% of insecticide residue concentrations (Keikotlhaile, Spanoghe et al. 2010, Liang, Liu et al. 2014). These findings demonstrate that to estimate insecticide exposure from questionnaires it is necessary to consider not just the types and amounts of foods that are consumed but also food preparation practices.

**4.2 TCPy**

In this study, chlorpyrifos was the only OP insecticide with a specific metabolite (TCPy) that was found above the limit of detection with a high frequency (89.3%). Chlorpyrifos residues are known to occur on fruits and vegetables in Australia ((FSANZ) Food Standards Australia New Zealand 2011). However, while increased consumption of fruits and vegetables was associated with higher TCPy concentrations, the association was not significant. This may be attributable to measurement error in the questionnaire, such as condensing all fruit and vegetable items into just two variables, despite the fact that chlorpyrifos concentrations may vary considerably between individual food items. Additionally, there may have been other unaccounted for sources of variation in TCPy concentrations. TCPy concentrations were associated with reported pest-spray use in the home and mouthing behaviours, suggesting a contribution from non-dietary sources of exposure to the observed variation in TCPy concentrations. Paradoxically, TCPy concentrations were higher when children with less frequent mouthing behaviour. It is possible that these associations are confounded by age. Chlorpyrifos is not available in any domestic spray-products in Australia, so the association between reported pest-spray use in the home and TCPy concentrations was unexpected. This finding may be due to chance or confounding. For example, households frequently using spray products may also use other chlorpyrifos containing products, such as some garden products, more frequently. Alternatively, some determinants of chlorpyrifos exposure may have been omitted from the exposure-assessment questionnaire. For example, elsewhere, chlorpyrifos concentrations in household dust have been found to correlate with reported termite and garden treatments at the home (Deziel, Colt et al. 2015). Furthermore, insecticides can persist in the indoor environment for years (Deziel, Ward et al. 2013). In this study, termite treatment was not specifically assessed, pest-control product use over only the past 12 months was assessed, and the sample size was too small to assess the association between reported garden insecticide use and biomonitoring data, which may explain why few questionnaire variables were found to be associated with TCPy concentrations.

**4.3 3-PBA**

Both dietary and several non-dietary variables were associated with pyrethroid metabolites, particularly 3-PBA, concentrations. In multivariable modelling, a relatively high amount of the total variability (77%) of 3-PBA concentrations was explained by these variables. Of the dietary variables, only vegetable intake was associated with 3-PBA concentrations. The relatively greater influence of non-dietary exposure factors may explain why, unlike the OPs, age was not associated with metabolite concentrations. Non-dietary variables associated with 3-PBA concentrations included frequency of domestic pest-spray product use in the summer months, season, a dog in the home, and frequency of hand-washing. Elsewhere, pest-product use at home has also been associated with increased concentrations of both organophosphate (Roca, Miralles-Marco et al. 2014) but particularly pyrethroid (Becker, Seiwert et al. 2006, Lu, Barr et al. 2006, Glorennec, Serrano et al. 2017) metabolites in children's urine, depending on the country, local regulations and therefore the insecticides commonly found in consumer pest-control products.

Despite the relatively small size of the study, we were able to assess the association of pest-spray product use and biomonitoring concentrations because of the relatively high frequency of use of these products. At least 40% of respondents had used a pest-control spray product in the past twelve months and 23% of participants used a pest-spray product at least weekly during the summer months. This frequency of use is similar to the relatively high frequency of pest-control product use reported in Florida USA (Naeher, Tulve et al. 2010) and higher than levels reported in the UK and other areas of the US (Grey, Nieuwenhuijsen et al. 2006, Guha, Ward et al. 2013). The similar high frequency of use may be attributable to the hot, humid climates in both Queensland and Florida associated with a higher pest burden. Insecticide exposure has previously been shown to vary seasonally, which has been attributed to seasonal variation in the availability of fresh fruits and vegetables as well as differences in frequency of application of domestic pest-control products (Wilson, Strauss et al. 2010, Food Standards Australia New Zealand 2011, Wu, Bennett et al. 2013). Insecticides that are applied in the domestic environment distribute to air and dust and are able to persist in the indoor environment (Colt, Lubin et al. 2004, Deziel, Colt et al. 2015).

Ongoing exposure of young children to insecticides that have been used in the domestic environment occurs predominantly via dermal absorption and non-dietary ingestion of household dust (Wilson, Strauss et al. 2010, Morgan 2012, Glorennec, Serrano et al. 2017). Other factors that may modify insecticide concentrations in dust or contact with dust may therefore also affect children's insecticide exposure. For example, the association between higher 3-PBA concentrations and the presence of a dog in the home may be explained by the fact that flea treatments and track-in of insecticides from outside the home by the dog lead to higher indoor dust insecticide concentrations (Lewis, Fortune et al. 2001, Becker, Seiwert et al. 2006, Morgan, Stout et al. 2008, Deziel, Ward et al. 2013). The association between increased hand-washing frequency and decreased 3-PBA biomonitoring concentrations observed in this study is likely due to increased hand-washing decreasing the duration and intensity of contact with insecticides in household dust.

**4.4 Strengths and limitations of the study**

The main strength of this study was the rigorous design and online format of the questionnaire. The questionnaire was designed following extensive literature reviews and primary research to identify insecticides that families are likely to be using in their homes, and the questionnaire was pre-tested prior to use, as previously described (English, Healy et al. 2015, English, Jagals et al. 2016, English, Chen et al. 2017). To minimise error associated with question interpretation, we used several visual cues to clarify pest-control related questions. We also included questions with visual cues about treatment of specific insects, to trigger participant recall of when pest-control products had last been applied. However, one of the main challenges with the design of the questionnaire was that data on insecticide use in Australia and human exposure pathways were relatively limited. As previously described, some important determinants of exposure may have been excluded from the questionnaire.

One of the main limitations of the study was that the questionnaire asked about behaviours over a period of weeks to months, whilst urine biomonitoring of insecticide metabolites only captures exposure in the order of hours to days (Nolan, Rick et al. 1984, Selim and Krieger 2007). Given that collecting urine samples from young children is practically difficult, parents were asked to collect just two urine samples from their child, although the ideal number for adequate exposure measurement is unknown (Attfield, Hughes et al. 2014). There was also an additional level of heterogeneity due to the study age group, since it included children pre and post-weaning. Limiting the study to children post-weaning may have reduced some of the variation. Because of the heterogeneity, it is likely that some associations have been attenuated towards the null.

Another limitation of urine biomonitoring in young children is the difficulty of standardising concentrations to account for differences in urinary dilution. Heffernan et al. described a urine flow method, which also has the advantage of enabling rapid calculation of estimated total daily intake, as well as excretion (Heffernan, Aylward et al. 2013). However, the urine flow model lacks sufficient parameter information for children in the age group in this study, and therefore could not be applied. Urinary excretion rates can also be calculated by multiplying the concentration of a contaminant in urine by the total volume of one urinary void and then dividing it by the time since the last void (Rigas, Okino et al. 2001). However, this is not practical in young children who are not toilet trained. Although creatinine is the most widely used method of standardising contaminant concentrations in urine, particularly in adult biomonitoring studies, the production of creatinine is more variable in young children (Barr, Wilder et al. 2005). We therefore presented summary results unadjusted and adjusted for creatinine and in the multivariable models creatinine was included as a covariate.

Another limitation of the study was the small sample size, which meant that some variables, such as organic food consumption, which is known to be an important determinants of dietary insecticide exposure (Oates, Cohen et al. 2014, Bradman, Quirós-Alcalá et al. 2015, Curl, Beresford et al. 2015, Berman, Göen et al. 2016, Glorennec, Serrano et al. 2017), could not be examined due to poor response distributions. Furthermore, some weak associations between exposure factors measured through the questionnaire and biomonitoring results may not have been detected and, conversely, some reported associations are likely to be spurious. Generalisability of the study findings are also limited to children residing in predominantly urban areas of South East Queensland.

**4.5 Future research directions and feasibility of the exposure-assessment questionnaire**

This study demonstrated that domestic pest-control practices and insecticide residues on food are likely to be the major contributors to young Australian children's insecticide exposure. However, more data in Australia are needed to better understand sources of insecticide exposure. Specifically, more data are needed on insecticide usage patterns and insecticide residues on food. These data would be informative to exposure risk assessment and the design of the exposure-assessment questionnaires.

In this study, the value of the questionnaire-based approach for identifying important determinants of exposure was demonstrated. Validation studies to determine the accuracy of the questionnaire-based approach to exposure assessment are warranted, given the utility that this approach would have for children's insecticide exposure assessment. Combining environmental data with the questionnaire-based approach also appears to be a promising approach, increasing the predictive capacity compared to using either tool alone. For example, matrices of the insecticides commonly found in pest-control products can be used to better estimate exposure to specific insecticides from pest-control products (Colt, Cyr et al. 2007), while combining food frequency questionnaire data with food surveillance data can improve dietary exposure estimates (Curl, Beresford et al. 2015, Chiu, Williams et al. 2018).

**5. Conclusion**

We have reported, for the first time, behavioural and dietary factors associated with biomarkers of insecticide exposure in Queensland infants and toddlers. Several factors were associated with insecticide metabolite concentrations, including age, diet, pets, mobility, hand-washing frequency, frequency of pest-product use in the home environment and season. Importantly, two of the questionnaire variables associated with insecticide metabolite concentrations are potentially modifiable, hand-washing and washing fruits and vegetables, suggesting that interventions to minimise children's insecticide exposure could be targeted at these behaviours. Further larger studies are required to assess the reproducibility of these findings and the generalisability to the broader Australian population.

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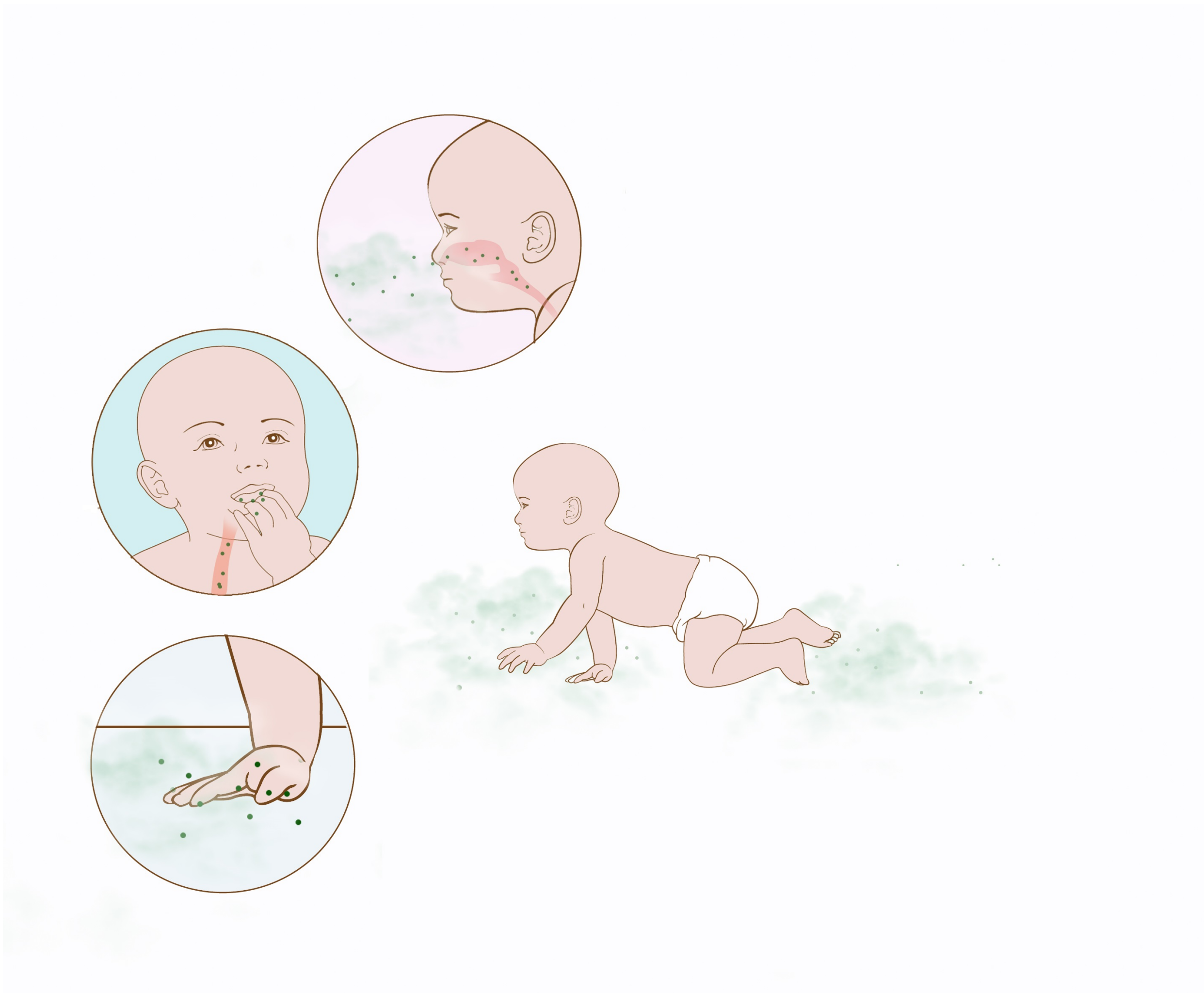

- Online questionnaire data was compared to kids urinary insecticide metabolites
- Significant dietary variables: fruit and vegetable intake and washing prior to eating
- Significant environmental factors: season and having a dog in the home.
- Significant behavioural factors: hand-washing and frequency of pest product use.
- Age was associated with organophosphate metabolite concentrations