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1 **EXPOSURES OF CHILDREN TO NEONICOTINOIDS IN PINE WILT DISEASE**
2 **CONTROL AREAS**

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25 **1. INTRODUCTION**

26 Agrochemicals, including insecticides and herbicides, have been used to protect agricultural
27 plants and forests from various kinds of pests, with general understanding on the balance of
28 costs and benefits in society. However, exposures to agrochemicals can induce acute and
29 chronic poisoning in sensitive populations, such as fetuses and infants as well as chemically
30 sensitive population at risk. In children, pesticide exposures reportedly occur via multiple
31 routes, including diet, drinking water, inhalation, and skin absorption (Chensheng et al. 2000).
32 Moreover, children living with parents who work with pesticides or who live in the proximity of
33 pesticide-treated farmlands are more susceptible to higher pesticide exposures than others living
34 in the same communities (Chensheng et al. 2000).

35 Children may be highly susceptible to the toxic effects of pesticides, with potential
36 developmental, dietary, and physiologic consequences (Roberts et al. 2012) that are exacerbated
37 by their rapid growth rates and high energy demands (caloric and oxygen requirements).
38 Compared with adults, children drink more water, eat more food, and breathe more air relative
39 to their body weights, likely facilitating the accumulation of high doses of pesticides in their
40 bodies (NRC 1993). In addition, blood–brain barriers of fetuses and neonates are immature
41 during brain development, allowing the passage and accumulation of various chemicals,

42 including pesticides, into fetal brains (Zheng et al. 2001). This may also result in higher
43 circulating levels and enhanced toxicity of pesticides in children (Weiss et al. 2004). Taken
44 together, the high susceptibility of children to pesticide exposures and the potential impacts of
45 these compounds on children's health are considered to be serious public and academic
46 concerns (CEH 2012).

47 Various insecticides, including organochlorines, organophosphates, and pyrethroids, have
48 been developed and widely used for pest management. However, insecticides that are less toxic
49 to humans relative to targeted species are highly sought, and since their introduction in the 1992,
50 neonicotinoids have been increasingly marketed. In 2008, neonicotinoids comprised 24% of all
51 marketed agrochemicals (total volume of €6.330 billion), mainly replacing organophosphates
52 (13.6%) and carbamates (10.8%) (Jeschke et al. 2011). Global neonicotinoid use has continued
53 to increase, and neonicotinoids have been registered for use in more than 120 countries, with a
54 total production volume of US\$2.5 billion across the globe (Akash et al. 2016). Neonicotinoids
55 were designed as specific agonist of insect nicotine-like receptors (nAChRs). However, recent
56 studies have shown that neonicotinoids can bind to not only insect nAChRs but also mammalian
57 nAChRs, with non-negligible dissociation constants (Kimura-Kuroda et al. 2012). These
58 receptors are of critical importance to human brain function, especially during the development
59 (Kandel et al. 2012) of memory, cognition, and behavior (Chen et al. 2014).

60 In Japan, pine trees are considered symbolic of the beauty of the natural environment and are
61 appreciated in mountain and coastal environments and in gardens of historic sites. However,
62 since the beginning of the 20th century, pine trees have been threatened by pine wilt disease in
63 Japan (Proença et al. 2017), and the nematode *Bursaphelenchus xylophilus* was identified as the

64 main cause. Because these worms are transmitted by the beetle species *Monochamus alternatus*
65 (Mamiya et al. 1988), pine trees have been protected from pine wilt by spraying insecticides
66 over large areas using helicopters or jet-spray machines, although effectiveness of such spraying
67 practices has remained to be proven.

68 Inhabitants of communities in such spraying zones are seriously concerned by these
69 practices, and complaints of health problems and symptoms that are related to pesticide toxicity
70 have been widely recorded. In previous studies, neonicotinoids were detected in urine samples
71 from Japanese women and children, and neonicotinoid concentrations in 3-year-old children
72 living in Aichi Prefecture of Japan ranged from the limit of detection (LOD) to 370 $\mu\text{g/g}$ of
73 creatinine, with a geometric mean of 4.16 $\mu\text{g/g}$ of creatinine (Ueyama et al. 2015; Osaka et al.
74 2016). However, despite the ubiquitous use of neonicotinoids in pine wilt control areas,
75 exposures and health impacts of neonicotinoids in these areas have not been formerly reported.
76 Thus, to assess neonicotinoid exposure levels of children (3 to 6 years old) living in Nagano
77 Prefecture of Japan, we determined concentrations of thiacloprid and other six neonicotinoids in
78 urine specimens and estimated daily exposure levels with regard to acceptable daily intake
79 (ADI) values.

80

81 **2. MATERIALS AND METHODS**

82 This study was approved by the Ethics Screening Committee of Hokkaido University (No.
83 28-1), and written informed consent was granted by all primary guardians prior to inclusion of
84 children in the study.

85 *2.1. Study areas*

86 Subjects were selected from communities in Nagano Prefecture, which is located in the
87 central part of Honshu Island, Japan. This prefecture has numerous valleys, high mountains and
88 forests, and the Japanese red pine (*Pinus densiflora*) is a predominant plant species in the local
89 forests, which are periodically sprayed with insecticides to control pine wilt disease.

90 2.2. *Study subjects*

91 Children of 3 to 6 years of age were recruited by advertising in city papers and in a local
92 newspaper, and a total of 46 (23 males and 23 females) subjects were included. Mean (range:
93 min. to max.) ages of male and female subjects were 4.8 (3 to 6) and 4.9 (3 to 6) years,
94 respectively. Early morning urine samples (before breakfast) were collected by guardians before
95 (May 26, 2016), during (June 23, 2016), and after (July 21, 2016) insecticide spraying events
96 using pre-distributed paper cups and were transferred into plastic 15 mL centrifuge tubes. Tubes
97 were then placed in zip lock bags and stored in household freezers until transfer to the
98 Department of Toxicology, Faculty of Veterinary Science, Hokkaido University, for analysis.

99 2.3. *Air Sample Collection*

100 Air samples were collected at two sites (A and B) in the proximity of the residences of the
101 studied subjects, and at a reference site in Suwa City, Nagano Prefecture, where neonicotinoids
102 have never been used to control pine wilt disease. Air samples (particulate matter) were
103 collected using low-volume air samplers equipped with quartz filters (2500QAT-UP 55 mm,
104 Pall life Sciences, Ann Arbor, USA). Air sampling was performed in affected areas from May
105 19 to 26, 2016, (2) from June 17 to 24, 2016, and (3) from July 14 to 22, 2016, and was
106 performed in the reference area on May 30, 2016, and June 6, 2016.

107 2.4. Analysis of neonicotinoid levels in urine specimens

108 Urine was thawed, stirred, and allowed to stand for 1 h. Neonicotinoids and their metabolites
109 were then extracted and purified using solid phase extraction methods. In these procedures,
110 Presep RPP cartridges (60 mg; Wako Pure Chemical Industries, Ltd., Osaka, Japan) were
111 conditioned with 2 mL of methanol and 2 mL of distilled water, and 1.0 mL aliquots of urine
112 were then thoroughly mixed with 5 ng (50 μ L of 100 ppb solution) of internal standards and
113 loaded into the cartridge. ENVIcarb/PSA (500 mg/300 mg; Sigma-Aldrich, Japan) cartridges
114 were then conditioned with 10 mL of acetone and connected in series with Presep RPP
115 cartridges and were then eluted with 8 mL of dichloromethane:acetonitrile (2:8,v/v) solution.
116 After concentrating and dry-solidifying using a centrifugal concentrator (CVE-200D with UT-
117 2000, EYELA, Tokyo, Japan), extracts were reconstituted with 100 μ L of 3% (v/v) methanol
118 solution and were transferred into vials for analysis.

119 The LC-ESI/MS/MS instrument (Shimadzu 20 A series with LCMS8040, Shimadzu Co.,
120 Kyoto, Japan) was equipped with a RSpak DE-213 (2 mm ID \times 150 mm) column (Showa
121 Denko, Tokyo, Japan) for sample analyses. HPLC solvents A and B comprised 0.1% (v/v)
122 formic acid and 10 mM acetic acid in water and 0.1% (v/v) formic acid and 10 mM acetic acid
123 in methanol, respectively, and were applied with the following gradient: $t = 0$ to 2 min, 20%
124 Solvent B; $t = 11$ min, 95% Solvent B; $t = 11$ to 13 min, 95% Solvent B. The column oven
125 temperature and flow rate were 45 $^{\circ}$ C and 0.4 mL/min, respectively. Multiple reaction
126 monitoring for mass spectrometry was programed as described in Table 1, and six
127 neonicotinoids and an acetamiprid metabolite, *N*-dm-acetamiprid, were detected in the range of
128 96% to 102%. Precision of analysis of all seven neonicotinoids was confirmed by multiple

129 analysis, with a relative standard deviation of 10% (Table 1). Analytes were quantitated using
130 internal standard methods, and calibration curves were generated for each analyte by mixing
131 compounds with blank urine specimens to final concentrations of 0.05, 0.1, 0.2, 0.5, 1.25, 2.5,
132 3.75, and 5 ng/mL. During the preparatory stage of our study, internal standards for nitenpyram
133 and N-dm-acetamid were not commercially available. Hence Nitenpyram was quantified
134 using the Dinotefuran internal standard; dindotefuran-d3. Our choice of Dinotefuran-d3 for
135 Nitenpyram quantification based on the similarity in the retention times Nitenpyram and
136 Dinotefuran, as well the similarities in physicochemical properties of both compounds. In the
137 absence of an internal standard, N-dm-Acetamid was also quantified using Acetamid-d6.
138 Undetectable neonicotinoid levels were confirmed in urine specimens from a volunteer who
139 mainly eats organic food, and these were then used as blank urine. Extraction and purification of
140 each calibration point was performed using the method described above, and linearity exceeds
141 $r^2 = 0.9$ in all calibration curves. Limits of quantitation (LOQs) were calculated as the lowest
142 points on standard curves (Table 1) with relative standard deviations of less than 15% ($n = 5$)
143 and signal-to-noise ratios of 5:1.

144 *2.5. Measurements of urinary creatinine concentrations*

145 Urinary creatinine concentrations were determined using Urinary Creatinine 10-Plate
146 Detection Kits (Arbor Assays, City, MI, USA) according to the manufacturer's instructions.

147 *2.6. Air sample preparation and analysis*

148 Particulate matter from study and reference areas were prepared as described previously
149 (Takenouchi et al. 2016). Briefly, shredded filters were put into 10 mL aliquots of ethyl acetate:
150 acetone (9:1,v/v) solutions and were sonicated for 5 min. Extracts were dried under a gentle

151 stream of nitrogen gas and were then dissolved in 1 mL of distilled water:acetone (4:1,v/v)
152 solution. Finally, extracts were filtered through 0.45 µm pore membrane filters (DISMIC-25cs,
153 Advantech, Tokyo, Japan) and were transferred into HPLC vials for LC-ESI/MS/MS analyses.

154 2.7. Calculation of EDI from urinary neonicotinoid concentrations

155 Estimated daily intake (EDI) of neonicotinoids was calculated from urinary neonicotinoid
156 and creatinine concentrations using the following formula:

$$157 \text{ EDI } (\mu\text{g/day}) = \text{urine neonicotinoids concentration } (\mu\text{g/g cre}) \times 0.3 \text{ (g cre/day)} \times 1/r \text{ (urinary} \\ 158 \text{ excretion correction coefficient)} \quad (1)$$

159 Excreted creatinine levels were assumed to be 0.3 g per day, as shown previously in children
160 (Sakurabayashi et al. 1999). Because kinetics parameters for thiacloprid have not been
161 established in humans, these were adopted from rat experiments ($r = 0.05$) in which 1.8% to
162 5.9% of orally ingested thiacloprid was excreted through urine (Pfeil et al. 2006). Kinetic
163 parameters for acetamiprid, clothianidin, dinotefuran, and imidacloprid in humans are reportedly
164 $r = 0.586, 0.596, 0.899,$ and $0.133,$ respectively (Harada et al. 2016). In the absence of
165 established correction coefficients for excretions of nitenpyram and thiamethoxam in humans,
166 we applied $r = 0.8$ and $0.6,$ respectively, for these neonicotinoids (JMPR 2010; FSC 2016).
167 Finally, daily exposures to thiacloprid and other neonicotinoids were calculated by applying
168 these coefficients to equation (1). According to a previously reported kinetic study (Harada et al.
169 2016), most of acetamiprid, once absorbed into the body, is rapidly metabolized and excreted to urine as
170 N-dm-acetamiprid. In the estimation of EDI values for acetamiprid, we incorporated N-dm-acetamiprid
171 data into acetamiprid data.

172 Atmospheric contributions to EDIs of neonicotinoids in children from study areas were
173 calculated using equation (2), with the assumption that the daily breathing volume of 1 to 12-
174 year-old children is 8.7 m³ (Kawahara et al. 2010):

$$175 \text{ Neonicotinoid intake from the atmosphere (ng/day)} = \text{Atmospheric neonicotinoid concentration} \\ 176 \text{ (pg/m}^3\text{)} \times \text{Daily breathing volume of children (8.7 m}^3\text{/day)} \quad (2)$$

177

178 2.8. Statistical analysis

179 Statistical analyses were performed using JMP 12 (SAS Institute Inc., Cary, NC, USA). Non-
180 parametric Steel–Dwass tests were performed to compare seasonal variations in urinary
181 neonicotinoid concentrations. Differences among groups were considered significant when
182 $p < 0.05$ in all analyses.

183

184 3. RESULTS AND DISCUSSION

185 3.1. Neonicotinoids in children's urine

186 Concentrations, detection frequencies, and percentiles of urinary neonicotinoids in children
187 living around target areas of thiacloprid (main ingredient of EcoOne-3 Flowable insecticide)
188 spraying are summarized in **Tables 2, 3 and 4**, and were calculated before (May), during (June),
189 and after (July) insecticide spraying was conducted. Frequencies of thiacloprid contents more
190 than LOQ in early-morning urine samples were **28%, 30%, and 33%** before, during, and after
191 insecticide spraying, respectively, and did not differ between sampling months (Tables 2, 3 and

192 4; Steel–Dwass test, $p > 0.05$). Moreover, no significant changes were observed in plots of
193 urinary thiacloprid concentrations against sampling times relative to insecticide spraying events
194 (Fig. 1). These analyses suggest that spraying of EcoOne-3-Flowable insecticide in target areas
195 of the pine wilt disease prevention program did not cause excessive thiacloprid exposures in
196 children. Alternatively, the days between insecticide spraying and urine sample collection may
197 have been sufficient for clearance of neonicotinoids. Accordingly, a previous study showed that
198 the first and terminal elimination phase half-lives of thiacloprid were 2.2 and 19.0 h in male rats
199 and 3.3 and 44.5 h in female rats, respectively (Pfeil et al. 2006). EcoOne-3-Flowable
200 insecticide was sprayed in the study area on the June 1 to 3, 9, 22, and 29 and urine samples
201 were collected on June 23, 2016, only 1 day after spraying. However, urinary thiacloprid
202 concentrations did not differ significantly before and after spraying, further suggesting that
203 thiacloprid is rapidly metabolized and eliminated. These observations indicate that exposures to
204 thiacloprid through EcoOne-3-Flowable insecticide spraying exercises are not commensurate
205 with absorbed levels in children.

206 In a similar recent study, Osaka and associates (Osaka et al. 2016) analyzed neonicotinoid
207 levels in urine samples from 3-year-old children (108 boys and 115 girls) living in Aichi
208 Prefecture, Japan, but did not detect thiacloprid in any of their samples. These discrepancies
209 with the present data may reflect their reported LOQ for thiacloprid (0.32 ng/mL), which was
210 much higher than our present LOQ of 0.05 ng/mL. Alternatively, Ueyama et al. (2015) observed
211 a steady increase in urinary thiacloprid detection frequencies in specimens that were collected
212 from adult women from 2003 to 2011, suggesting that the present data reflect an increasing
213 trend of neonicotinoid use in Japan.

214 In addition to thiacloprid, six other neonicotinoid compounds that are not constituents of
215 EcoOne-3-Flowable insecticides were detected in the children of this study (Tables 2, 3 and 4).
216 Although acetamiprid was detected in only 8.5% to 12.8% of subjects, its major metabolite *N*-
217 dm-acetamiprid was present in 86.6% to 93.5% of our urine specimens. Detection frequencies of
218 the other neonicotinoids were 28.3% to 46.6% for thiamethoxam, 43.4% to 54.3% for
219 dinotefuran, and 41.3 to 52.2% for clothianidin. These detection frequencies in children were far
220 higher than those of thiacloprid (Tables 2, 3 and 4), and absolute urinary concentrations of these
221 neonicotinoids were higher than those of thiacloprid (Table 2, 3 and 4; Fig. 2).

222 Urinary concentrations of nitenpyram increased from May (below LOQ) to June (10.3 µg/L;
223 Tables 2 and 3, Fig. 1), suggesting that nitenpyram exposures follow the consumption of
224 agricultural products. Although pesticide use in rice, fruit, and vegetables from the study area
225 usually increase during June, the precise contributions of domestic and international agricultural
226 products to nitenpyram exposures in children remain unknown. In contrast, urinary
227 concentrations of dinotefuran, thiacloprid, *N*-dm-acetamiprid, and clothianidin did not differ
228 significantly during sampling periods (Tables 2, 3 and 4; Fig. 1), indicating that exposures of the
229 children to neonicotinoids occur at the present study areas irrespective of the timing of spraying
230 of thiacloprid. Hence further studies are needed to identify exact exposure sources of
231 neonicotinoids in the present study area.

232 Children seemed to be exposed to multiple kinds of neonicotinoids in June and July compared to
233 May, the observation of which may reflect a more active pest control activity in summer in this
234 area. Generally, the majority (more than 80%) of the children were found to be exposed to

235 multiple kinds of neonicotinoids in the study areas (Fig. 2), warranting investigations of
236 synergistic effects of neonicotinoids in these children.

237

238 3.2. Atmospheric neonicotinoids

239 Neonicotinoid concentrations in atmospheric particulate matter from sites A and B are
240 presented in Table 5. Among these, atmospheric thiacloprid concentrations were 67.9, 25.8, and
241 35 pg/m^3 before, during, and after spraying of EcoOne-3-Flowable insecticide (Table 5),
242 respectively. The comparatively high levels before spraying preclude associations with EcoOne-
243 3-Flowable insecticide spraying activities in this study, although our air sampling was
244 performed 14 days after spraying of the EcoOne-3-Flowable insecticide, allowing residual
245 thiacloprid to diffuse away before the sampling period. In contrast, atmospheric concentrations
246 of thiacloprid in site B were 90 pg/m^3 during the spraying exercise, were only 32 pg/m^3 before
247 spraying, and were 45 pg/m^3 after spraying. Hence, the elevated levels of thiacloprid observed
248 in June likely follow variations in atmospheric thiacloprid concentrations. Similarly, Takenochi
249 et al. (2016) collected air samples during a similar spraying period at the Togura region in
250 Chikuma City of Nagano Prefecture in 2013. Their determinations of thiacloprid concentrations
251 in atmospheric particulate matter showed increased thiacloprid concentrations from below LOQ
252 ($<35 \text{ pg}/\text{m}^3$) to 1,900 pg/m^3 immediately after spraying exercises and restoration of baseline
253 thiacloprid levels below the LOQ after only 1 day. These observations confirm that thiacloprid
254 has a short atmospheric residence time, presumably due to its low vapor pressure (Table S2).
255 The disparity between the trends of atmospheric thiacloprid concentrations observed in the
256 report by Takenochi et al. (2016) and that of the present study may be due to differences in

257 sampling spots. Whereas Takenochi. et al. (2016) collected atmospheric particulate matter
258 beside the sprayed spots, samples in the present study were collected in the residential area a
259 few kilometer far from the sprayed spots. Apparently, a greater proportion of the thiacloprid
260 concentrations in the atmosphere might have either drifted away or settled on the ground as at
261 the time of sampling for the present study.

262

263 3.3. EDI of neonicotinoids

264 EDI of thiacloprid in children from the study areas were maximal at 2.15 $\mu\text{g}/\text{day}$, and 75th
265 percentile amounts of intake were 0.287, 0.310, and 0.367 $\mu\text{g}/\text{day}$ in May, June, and July,
266 respectively (Table 6,7 and 8). These amounts are within the ADI of 12 $\mu\text{g}/\text{kg}/\text{day}$, which is
267 equivalent to 180 $\mu\text{g}/15 \text{ kg}/\text{day}$ in children (Table S1).

268 In addition, we compiled EDI values of other neonicotinoids besides thiacloprid in children
269 during the EcoOne-3-Flowable insecticide spraying exercise in June (Table 7). At an EDI of
270 15.2 $\mu\text{g}/\text{kg}/\text{day}$, daily intake of acetamiprid was about 1.4% of its acceptable daily intake (ADI),
271 whereas dinotefuran (31.4 $\mu\text{g}/\text{kg}/\text{day}$) was consumed at 1.0% of its ADI, nitenpyram
272 (8.92 $\mu\text{g}/\text{kg}/\text{day}$) was consumed at 0.1% of its ADI, and thiamethoxam (0.813 $\mu\text{g}/\text{kg}/\text{day}$) was
273 consumed at 0.3% of its ADI (Table 7). Although these EDIs are relatively low compared to
274 ADIs, the maximal EDI of 51.6 $\mu\text{g}/\text{kg}/\text{day}$ during the pesticide spraying season suggests that the
275 EcoOne-3-Flowable spraying coincided with other agricultural activities that increased
276 exposures of children to these neonicotinoids. The maximum EDI of imidacloprid in children
277 was 11.1 $\mu\text{g}/\text{day}$ (1.3% of its ADI) before the EcoOne-3-Flowable insecticide spraying exercise
278 (Table 6) but was 1.23 $\mu\text{g}/\text{kg}/\text{day}$ in June (0.1% of ADI; Table7) and 3.60 $\mu\text{g}/\text{kg}/\text{day}$ (0.4% of

279 ADI; Table 8) in July, indicating limited effects of the spraying on imidacloprid exposure levels
280 among our study subjects. Moreover, whereas peak EDI for acetamiprid and imidacloprid were
281 greater than those of all the other detected neonicotinoids, these did not exceed 2% of ADI
282 (Tables 6, 7 and 8).

283 Finally, we determined contributions of neonicotinoid inhalation from the atmosphere and
284 showed that inhaled thiacloprid amounts were between 0.22 and 0.78 ng/day in the study area
285 (Site A and B, Table 9), which are less than 1% of the EDI of thiacloprid (maximum
286 0.516 µg/day). We also found that inhalation from the atmosphere contributed very little to
287 other neonicotinoid exposures (Table 9). Generally, neonicotinoids have very low vapor
288 pressure (Raina-Fulton 2016; Table S2) meaning that most neonicotinoid compounds, especially
289 imidacloprid and thiacloprid have limited volatility and short residence time in the atmosphere.
290 Hence it is possible that thiacloprid which was used for the aerial spraying exercise in the
291 present study area, quickly settled on soil and/or water immediately after spraying exercise, and
292 thence limited inhalation among the children. Collectively, these data suggest that ingestion of
293 neonicotinoids from foods and drinks contributes predominantly to total intakes by children and
294 that inhaled neonicotinoid exposures are very limited in the present study areas.

295 EDIs of all detected neonicotinoids in the children of the present study were far lower than
296 ADI values. However, a recent study indicates that exposures to no obvious adverse effect levels
297 (NOAELs) of neonicotinoids may induce adverse effects in animals. Specifically, whereas the
298 NOAEL of clothianidin has been set at 9.7 mg/kg (NRDC 2016), 5 mg/kg clothianidin
299 reportedly induced anxiety-related behaviors in mice (Hirano et al. 2018), suggesting that the
300 accepted NOAEL for clothianidin should be revised to a lower threshold. In the absence of

301 robust evidence, a clothianidin NOAEL value of 0.5 mg/kg (one-tenth of 5 mg/kg) would
302 correspond with an ADI value of 0.005 mg/kg per day (using 100 as an uncertainty factor and
303 15 kg as the child's body weight). Under these conditions, the present clothianidin EDI of
304 7.6 µg/kg/day represents about 10% of the ADI. In another study, Sun et al. (2016) reported that
305 exposures to daily imidacloprid doses of 0.06 mg/kg promoted high-fat-induced adiposity and
306 insulin resistance in male mice. These observations also imply that at 1% of the NOAEL
307 (5.7 mg/kg), imidacloprid may affect energy metabolism via the AMP-activated protein kinase-
308 α pathway. In the present study, the NOAEL for imidacloprid that we used was 0.006 mg/kg,
309 which was one-tenth of 0.06 mg/kg in Sun et al.'s study; therefore, on comparing the results, the
310 EDI of imidacloprid in the present children was two times higher than the ADI (currently 1.3%
311 of the ADI). Hence, further studies are warranted to precisely assess the toxicity of
312 neonicotinoids in humans and adjust ADI values accordingly.

313

314 **4. CONCLUSIONS**

315 In this study, concentrations of the neonicotinoids acetamiprid, clothianidin, dinotefuran,
316 imidacloprid, nitenpyram, thiacloprid, and thiamethoxam were determined in urine samples
317 from children living in areas where thiacloprid was used to control pine wilt disease. Subsequent
318 analyses showed very limited neonicotinoid inhalation among children. However, the presence
319 of six other neonicotinoids reflected high intakes of agricultural products by these children,
320 although estimated intake levels of neonicotinoids were less than 2% of ADI values. Finally,
321 whereas current exposure levels of the compounds detected in this study were far below the
322 doses that induce acute toxicity, sufficient caution should be taken to avoid the potential

323 cumulative impacts of these compounds in sensitive populations, especially among children and
324 chemically sensitive individuals.

325

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333 **Conflicts of interest**

334 The authors declare no conflicts of interest.

335 **Data Accessibility**

336 Data, associated metadata, and calculation tools are available by contacting the corresponding
337 author (y_ikenaka@vetmed.hokudai.ac.jp; Tel: +81-11-706-5102; Fax: +81-11-706-5105
338).

339

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413 **Figure Captions**

414 Fig.1: Time course of Thiachloprid and other neonicotinoid concentrations in urine ($\mu\text{g/g Cre}$);
415 Time course plots did not include urinary neonicotinoid concentrations below the limits of
416 quantification.

417 Fig.2: Multiple exposure evaluation of neonicotinoids among the children; most children were
418 found to be exposed to multiple neonicotinoid compounds.

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432 Table 1: Selected neonicotinoids and their metabolites

Target Neonicotinoids	LOQ* (ppb)	Recovery rate (%)	RSD** (%)	MRM***	Polarity for ESI
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Acetamiprid	0.05	102	7	223.0 > 126.0	+
Clothianidin	0.1	92	4	249.0 > 132.1	+
Dinotefuran	0.1	100	8	203.0 > 129.1	+
Imidacloprid	0.2	100	5	256.0 > 209.1	+
Nitenpyram	0.1	98	5	271.0 > 126.1	+
Thiacloprid	0.05	100	5	252.9 > 126.1	+
Thiamethoxam	0.1	96	4	291.9 > 211.0	+
<i>N</i> -dm- Acetamiprid	0.05	101	9	208.9 > 126.1	+

Internal Standards

Acetamiprid d6	-	-	4	229.0 > 126.0	+
Clothianidin d3	-	-	4	249.0 > 132.1	+
Dinotefuran d3	-	-	7	206.0 > 132.3	+
Imidacloprid d4	-	-	10	259.7 > 179.2	+
Thiacloprid d4	-	-	6	256.9 > 126.1	+
Thiamethoxam d4	-	-	4	295.7 > 215.1	+

433 * LOQ = limit of quantification, ** RSD = relative standard deviation; *** MRM = multiple
434 reaction monitoring; ESI = electrospray ionization

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Table 2 : Urinary neonicotinoids ($\mu\text{g/L}$) in children before the insecticide spraying in May 26, 2016.

Neonicotinoid	Frequency (%)	Selected percentile				
		25th	50th	75th	95th	Max
Acetamiprid	9	<LOD	<LOD	<LOD	0.21	0.53
Clothianidin	41	<LOD	<LOD	0.50	1.32	3.60
Dinotefuran	43	<LOD	<LOD	0.55	10.25	15.60
Imidacloprid	13	<LOD	<LOD	<LOD	0.35	4.70
Nitenpyram	0	<LOD	<LOD	<LOD	<LOQ	<LOQ
Thiacloprid	28	<LOD	<LOD	0.06	0.06	0.10
Thiamethoxam	28	<LOD	<LOD	0.10	0.26	0.85
<i>N</i> -dm-Acetamiprid	91	0.27	0.39	0.71	3.48	11.16
Σ NEO	98	0.59	1.34	2.58	12.38	16.06

Max = Maximum; LOD = limit of detection, NEO = neonicotinoid.

Table 3 : Urinary neonicotinoids ($\mu\text{g/L}$) in children during the insecticide spraying exercise (June 23, 2016).

Neonicotinoid	Frequency (%)	Selected percentile				
		25th	50th	75th	95th	Max
Acetamiprid	11	<LOQ	<LOQ	<LOQ	0.27	0.54
Clothianidin	52	<LOQ	0.14	0.59	3.18	4.58
Dinotefuran	54	<LOQ	0.14	0.68	5.13	72.31
Imidacloprid	15	<LOQ	<LOQ	<LOQ	0.36	0.64
Nitenpyram	30	<LOQ	<LOQ	0.20	2.23	10.83
Thiacloprid	30	<LOQ	<LOQ	0.06	0.06	0.13
Thiamethoxam	37	<LOQ	<LOQ	0.12	0.67	1.71
<i>N</i> -dm-Acetamiprid	93	0.25	0.34	0.66	5.09	7.17
Σ NEO	100	0.59	1.79	4.13	10.85	75.09

All abbreviations have been defined in Table 2.

Table 4 : Urinary neonicotinoids ($\mu\text{g/L}$) in children after the insecticide spraying exercise (July 21, 2016).

Neonicotinoid	Frequency (%)	Selected percentile				
		25th	50th	75th	95th	Max
Acetamiprid	11	<LOQ	<LOQ	<LOQ	0.23	1.34
Clothianidin	49	<LOQ	<LOQ	0.64	3.24	6.02
Dinotefuran	49	<LOQ	<LOQ	0.21	1.54	8.58
Imidacloprid	18	<LOQ	<LOQ	<LOQ	0.39	1.48
Nitenpyram	27	<LOQ	<LOQ	0.19	0.26	0.63
Thiacloprid	33	<LOQ	<LOQ	0.06	0.06	0.10
Thiamethoxam	47	<LOQ	<LOQ	0.13	0.47	1.92
<i>N</i> -dm-Acetamiprid	87	0.24	0.46	0.98	6.42	18.72
Σ NEO	100	0.59	1.14	2.68	11.79	19.33

All abbreviations have been defined in Table 2.

Table 5: Atmospheric neonicotinoid concentrations (pg/m^3) in dust from sites A and B and the control site (EcoOne-3-Flowable Non-Spraying Area)

Neonicotinoid	Site A			Site B			Control Site
	5/19- 5/26	6/17- 6/24	7/14- 7/22	5/19- 5/26	6/17- 6/24	7/14- 7/22	5/30- 6/6
Acetamiprid	54.2	77.5	35.5	58.3	60.6	47.5	43.6
Clothianidin	26.1	<LOQ	<LOQ	50.2	<LOQ	5.1	<LOQ
Dinotefuran	<LOQ	<LOQ	128.9	76.5	71.3	216.7	<LOQ
Imidacloprid	98.8	76.0	56.1	143.1	100.5	52.5	64.4
Nitenpyram	<LOQ	<LOQ	<LOQ	0.2	1.6	<LOQ	<LOQ
Thiacloprid	67.9	25.8	35.0	32.1	90.0	45.3	18.9
Thiamethoxam	64.2	54.2	44.7	<LOQ	<LOQ	44.2	<LOQ
<i>N</i> -dm-Acetamiprid	7.9	7.0	6.1	8.3	5.8	6.3	6.8

<LOQ = below the limit of detection.

Table 6: Estimated daily intake (EDI) of neonicotinoids in children before insecticide spraying exercise (26th May)

Neonicotinoid	EDI percentile ($\mu\text{g}/\text{day}$)				Max	%ADI
	25th	50th	75th	95th		
Acetamiprid	0.145	0.393	0.568	5.37	9.35	0.9
Clothianidin	-	-	0.298	1.02	1.99	0.1
Dinotefuran	-	-	0.230	9.22	15.90	0.5
Imidacloprid	-	-	-	1.05	11.0	1.3
Nitenpyram	-	-	-	-	-	-
Thiacloprid	-	-	0.287	1.29	2.15	1.2
Thiamethoxam	-	-	0.030	0.201	0.407	0.2
ΣNEO	0.618	1.02	2.40	12.5	19.1	0.1

Max = maximum EDI of neonicotinoids among subjects; %ADI = percent of acceptable daily intakes. EDI values below limits of quantification are indicated by “-”. Estimated exposures were calculated assuming that creatinine excretion in children is 0.3 g per day. The excretion coefficients “r” of Acetamiprid, Clothianidin, Imidacloprid and Dinotefuran were retrieved from Harada et al. 2016 ($r = 0.586, 0.596, 0.133, \text{ and } 0.899$, respectively). Excretion coefficients “r” of Nitenpyram, Thiacloprid, and Thiamethoxam were inferred from animal experiments (thus; 0.8, 0.05 and 0.6 for Nitenpyram, Thiacloprid, and Thiamethoxam respectively).

Table 7: Estimated daily intake (EDI) of neonicotinoids in children during insecticide spraying exercise (23rd June)

Neonicotinoid	EDI percentile ($\mu\text{g}/\text{day}$)				Max	%ADI
	25th	50th	75th	95th		
Acetamiprid	0.410	0.240	0.521	5.17	15.2	1.4
Clothianidin	-	0.086	0.318	1.25	3.64	0.2
Dinotefuran	-	0.033	0.240	1.80	31.4	1.0
Imidacloprid	-	-	-	0.961	1.23	0.1
Nitenpyram	-	-	0.094	1.95	8.92	0.1
Thiacloprid	-	-	0.310	0.796	1.77	1.0
Thiamethoxam	-	-	0.065	0.408	0.813	0.3

ΣNEO	0.486	1.26	3.08	10.3	51.6	0.3
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All abbreviations have been defined in Table 6.

Table 8: Estimated daily intake (EDI) of neonicotinoids in children after insecticide spraying exercise (21st July)

Neonicotinoid	EDI percentile (µg/day)				Max	%ADI
	25th	50th	75th	95th		
Acetamiprid	0.153	0.307	0.904	3.05	13.7	1.3
Clothianidin	-	-	0.418	1.98	7.59	0.5
Dinotefuran	-	-	0.136	0.611	3.16	0.1
Imidacloprid	-	-	-	1.87	3.60	0.4
Nitenpyram	-	-	0.057	0.208	0.468	0.0
Thiacloprid	-	-	0.367	0.811	1.22	0.7
Thiamethoxam	-	-	0.060	0.376	1.01	0.4
ΣNEO	0.565	1.13	2.97	8.29	19.4	0.1

All abbreviations have been defined in Table 6.

Table 9: Daily atmospheric neonicotinoid exposure estimates (ng/day) in children

Neonicotinoid	Site A			Site B		
	May	June	July	May	June	July
Acetamiprid	0.47	0.67	0.31	0.51	0.53	0.41
Clothianidin	0.23	-	-	0.44	-	0.04
Dinotefuran	-	-	1.12	0.67	0.62	1.88
Imidacloprid	0.86	0.66	0.49	1.24	0.87	0.46
Nitenpyram	-	-	-	0.00	0.01	-
Thiacloprid	0.59	0.22	0.30	0.28	0.78	0.39
Thiamethoxam	0.56	0.47	0.39	-	-	0.38
<i>N</i> -dm-Acetamiprid	0.07	0.06	0.05	0.07	0.05	0.05

Neonicotinoid intake from the atmosphere (ng/day) = Atmospheric neonicotinoid concentration (pg/m³) × Child's daily breathing volume 8.7 m³/day. The daily breathing volume in children of 1–12 years of age was assumed as 8.7 m³ (Koenig et al. 2000). Daily atmospheric exposures below limits of quantification are indicated by “-”.

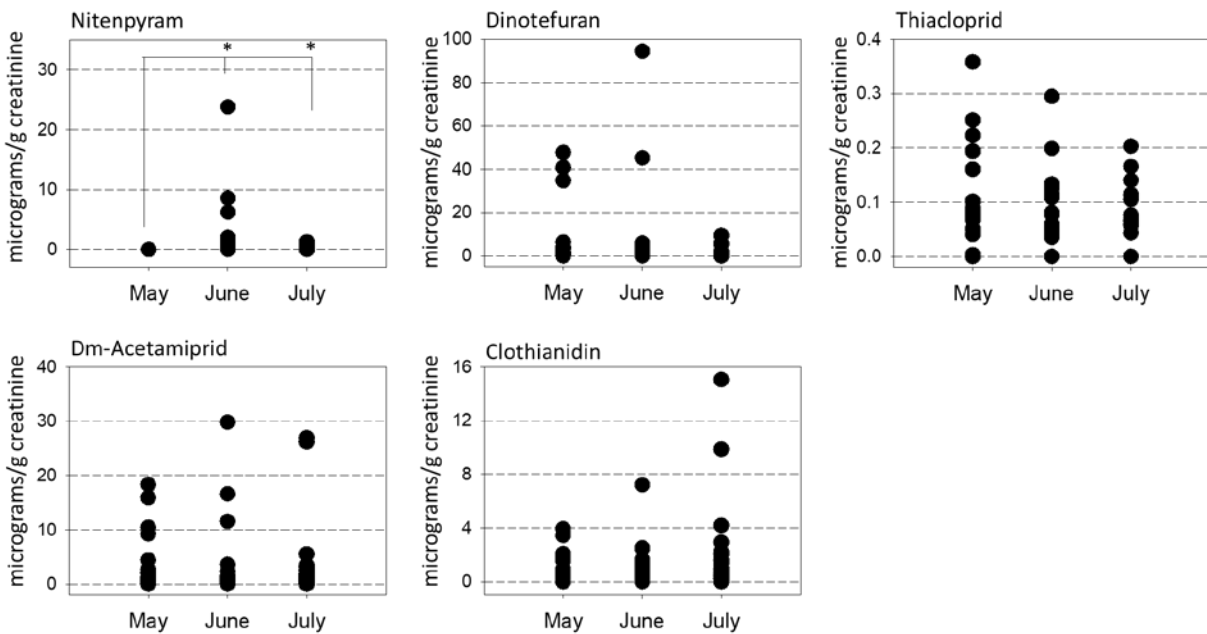


Fig.1: Time course of Thiachloprid and other neonicotinoid concentrations in urine ($\mu\text{g/g Cre}$); Time course plots did not include urinary neonicotinoid concentrations below the limits of quantification.

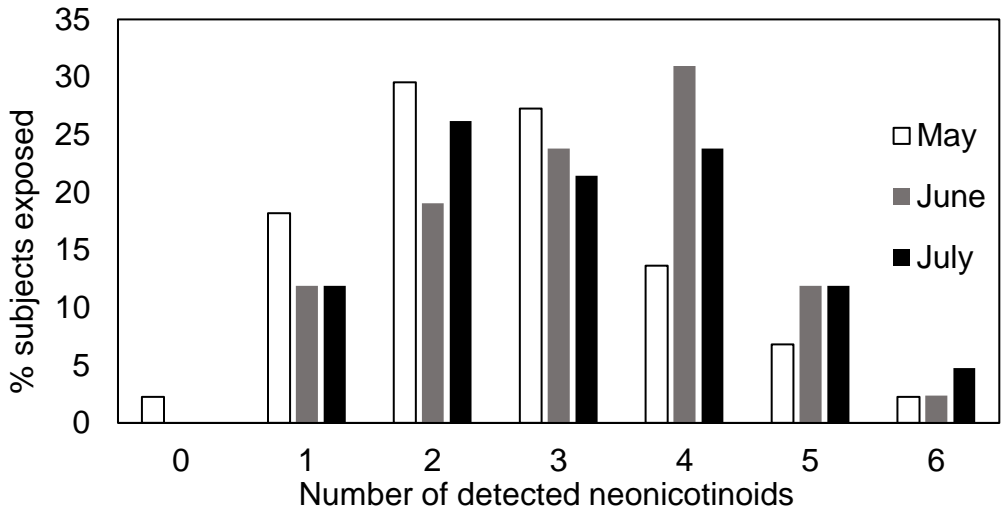


Fig.2: Multiple exposure evaluation of neonicotinoids among the children; most children were found to be exposed to multiple neonicotinoid compounds.

Reference data

Table S1: Maximum residual limits (MRL) and acceptable daily intakes (ADI) of neonicotinoids

Neonicotinoid	MRL for Tea*mg/kg	MRL for Spinach* mg/kg	MRL for Strawberry* mg/kg	ADI mg/kg/day
Acetamiprid	30	3	3	0.071
Clothianidin	50	40	0.7	0.097
Imidacloprid	10	15	0.5	0.057
Nitenpyram	10	5	5	0.53
Thiacloprid	30	-	5	0.012
Thiamethoxam	20	10	2	0.018
Dinotefuran	25	15	2	0.22

* The Japan Food Chemical Research Foundation (2016)

Table S2: Physicochemical properties of neonicotinoids

Neonicotinoid	Water Solubility (mg/L)	Vapor Pressure (Pa)	Log Kow	Soil Half-Life (days)
Acetamiprid	4,250 (25°C)	1×10^{-6} (25°C)	0.80	3 (4–7)
Clothianidin	327 (20°C)	1.3×10^{-10} (25°C)	0.91	545 (13–1,386)
Imidacloprid	610 (20°C)	4×10^{-10} (25°C)	0.57	191 (104–228)
Nitenpyram	600,000 (20°C)	$<1 \times 10^{-4}$ (50°C)	-0.64	-
Thiacloprid	185 (20°C)	8×10^{-10} (25°C)	1.26	15.5 (9–27)
Thiamethoxam	4,100 (25°C)	2.7×10^{-9} (20°C) 6.6×10^{-9} (25°C)	-0.13	50 (7–72)
Dinotefuran	39,830 (25°C)	$<1.7 \times 10^{-6}$ (30°C)	-0.55	82

Raina-Fulton R. 2016.<http://www.avidscience.com/wp-content/uploads/2016/08/PST>

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