Environmental Toxicology

Estimation of the Effects of Neonicotinoid Insecticides on Wild Raccoon, *Procyon lotor*, in Hokkaido, Japan: Urinary Concentrations and Hepatic Metabolic Capability of Neonicotinoids

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Abstract: Toxicological effects of neonicotinoid insecticides (NNIs) have been reported for mammals, such as humans, rats, and mice. However, there are limited reports on their toxic effects on wild mammals. To predict NNI-induced toxic effects on wild mammals, it is necessary to determine the exposure levels and metabolic ability of these species. We considered that raccoons could be an animal model for evaluating NNI-induced toxicities on wildlife because they live near agricultural fields and eat crops treated with NNIs. The objective of the present study was to estimate the effects of NNI exposure on wild raccoons. Urinary concentrations of NNI compounds ($n = 59$) and cytochrome P450-dependent metabolism of NNIs ($n = 3$) were evaluated in wild raccoons captured in Hokkaido, Japan, in 2020. We detected either one of the six NNIs or one metabolite, including acetamiprid, imidacloprid, clothianidin, dinotefuran, thiacloprid, thiamethoxam, and desmethyl-acetamiprid in 90% of raccoons (53/59); the average cumulative concentration of the seven NNI compounds was 3.1 ng/ml. The urinary concentrations were not much different from those reported previously for humans. Furthermore, we performed an in vitro assessment of the ability of raccoons to metabolize NNIs using hepatic microsomes. The amounts of NNI metabolites were measured using liquid chromatography–electrospray ionization–tandem mass spectrometry and compared with those in rats. Raccoons showed much lower metabolic ability; the maximum velocity/Michaelis–Menten constant (V_{max}/K_m) values for raccoons were one-tenth to one-third of those for rats. For the first time, we show that wild raccoons could be frequently exposed to NNIs in the environment, and that the cytochrome P450-dependent metabolism of NNIs in the livers of raccoons might be low. Our results contribute to a better understanding of the effects of NNIs on raccoons, leading to better conservation efforts for wild mammals. *Environ Toxicol Chem* 2022;41:1865–1874. © 2022 The Authors. *Environmental Toxicology and Chemistry* published by Wiley Periodicals LLC on behalf of SETAC.

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INTRODUCTION

Wild mammals are exposed to various environmental pollutants worldwide. Insecticides are among the most influential xenobiotics that have harmful effects on animals. Several negative effects on wild mammals of persistent and highly toxic insecticides such as organochlorines and organophosphates have been reported in the past (Blus, 2011; Grue et al., 1997; Kwon et al., 2004; Herceg Romanić et al., 2015). However, the

use of such compounds has decreased (Van Den Berg et al., 2017). Recently, the neonicotinoid insecticides (NNIs) have been the subject of more attention in the field of wildlife toxicology (Gibbons et al., 2015; Pisa et al., 2021). The NNIs were developed and commercialized in the early 1990s and rapidly became some of the most frequently and widely used insecticides worldwide, in agriculture, veterinary medicine, and the residential environment (Simon-Delso et al., 2015). The NNIs are nicotinic acetylcholine receptor agonists with insect-selective activity and are designed to have low toxicity in mammals (Simon-Delso et al., 2015; Wang et al., 2018). Because of these advantageous characteristics, NNIs account for nearly one-fifth of the world's insecticide shipments (Wang et al., 2018). Various types of NNIs, including acetamiprid, imidacloprid, clothianidin, dinotefuran, thiacloprid, thiamethoxam, and nitenpyram, have been registered in more than 120 countries mainly for agricultural use (Casida, 2018). In Japan, NNIs are some of the most abundantly used insecticides, with annual shipments amounting to more than 400 tons in 2020 (National Institute for Environmental Studies, 2022).

Wildlife species are exposed to NNIs in the environment and are reported to have many toxic effects. For example, exposure to low doses of NNIs is believed to cause more mortality in bee species compared with that in target insects, and exposure to NNIs is possibly one of the important factors in colony collapse disorder (Lundin et al., 2015; Whitehorn et al., 2012). In the aquatic ecosystem of a lake in Japan, the application of NNIs coincided with a decrease in average zooplankton biomass and caused a deterioration in the smelt harvest (Yamamuro et al., 2019). In avian species, exposure to less than the lethal dose of NNIs disrupted their migratory behavior (Eng et al., 2019). Humans are also exposed to NNIs through the oral route after consumption of agricultural products. (Ikenaka et al., 2019; Nimako et al., 2021) A possible relationship between chronic NNI exposure and neurological disorders, such as attention-deficit hyperactivity disorder, has been suggested (Roberts et al., 2019).

The NNI-induced toxicological effects on animals mainly depend on two factors: the exposure dose and the susceptibility to the chemical. There are some reports regarding NNI detection in wild mammalian species (Byholm et al., 2018; Kaczyński et al., 2021; MacDonald et al., 2018). In these previous studies, NNIs were detected in organs such as the liver and muscle of certain wildlife species like wild boar, deer, and birds; however, the rate of detection and concentrations were low in most cases, and exposure levels of NNIs cannot be estimated from the concentrations in these organs. In a pharmacokinetic analysis, more than 60% of the NNIs were found to be excreted through urine in mice, and concentrations of NNI residues in urine were much higher than those in organs or tissues (Ford & Casida, 2006). In a human study, the urinary excretion correction coefficient was used to estimate exposure amounts of NNIs (Ikenaka et al., 2019). Therefore, urinary samples are the most appropriate for accurate estimation of the rate of exposure and exposure amounts of NNIs in wild mammals. In addition, susceptibility to NNIs in wild mammals has not been investigated (Pisa et al., 2021). The NNIs are metabolized mainly in the liver by cytochrome P450 (CYP), which is a phase I xenobiotic-metabolizing enzyme (Shi et al., 2009). The metabolic ability of CYP is an important factor in determining the susceptibility of an animal species to NNIs (Khidkhan et al., 2021). To assess NNI-induced toxicities, animal species with a high toxicological risk, such as highly sensitive species, should be used to avoid underestimating the toxic effects.

In the present study, we focused on the raccoon, *Procyon lotor*, as an animal model to evaluate the NNI-induced effects on wild mammals. They have been reported to inhabit suburban agricultural areas and forest areas in Hokkaido, Japan, and they eat crops (Osaki et al., 2019). Although there are no previous reports about the NNI exposure situation in raccoons, they could be exposed to NNIs after eating sprayed crops. In addition, in the Canidae family, the metabolic abilities of CYPs are generally lower than in other mammals such as rats (Ishizuka et al., 2006). Raccoons are members of the Procyonidae species, included in the Canidae family, and may have a lower ability for CYP-dependent NNI metabolism than other species. Owing to the possibilities of high exposure risk and low metabolic abilities, we surmised that raccoons could be used as indicator animals for evaluating the toxicological effects of NNIs on wild mammalian species. Raccoons were imported into Japan as companion animals from North America in the 1980s and are now distributed throughout the country as an invasive alien species (Ikeda et al., 2004). Therefore, raccoons have been euthanized in control programs to conserve the ecosystem, and we were able to obtain several samples of these euthanized individuals.

In the present study, we measured the urinary concentrations of NNIs to determine the exposure of wild raccoons in Hokkaido, and we investigated their hepatic metabolic ability, to evaluate their susceptibility to these compounds.

MATERIALS AND METHODS

Animals and treatments

Using box trap systems (Havahart Large Collapsible Pro Cage Model 1089; Woodstream), 54 raccoons were captured in 2021 from a forest surrounded by cropland in Hokkaido. Raccoons were collected from April to October when crops are generally cultivated outside, and NNIs are thought to be applied to crops; thus, the sampling period was considered to be optimal for the assessment of NNI exposure to raccoons. The captured raccoons were anesthetized in the box traps with 1.2 mg/kg butorphanol tartrate (Vetorphale; Meiji Seika), 40 µg/kg medetomidine hydrochloride (Dolbene; Kyoritsu), and 0.2 mg/kg midazolam (Dormicum; Astellas) administered through an intramuscular injection. Then the raccoons were euthanized by injecting potassium chloride into their hearts. Another five carcasses were collected as part of the feral raccoon control program in Hokkaido, for a total of 59 to be used in our study. Raccoon body weights and sex were recorded, and age was estimated using skulls (Junge & Hoffmeister, 1980). The age, sex, and date of capture are presented in the Supporting

Information, Table S1. Urine samples were collected by cystocentesis and kept at −20 °C until the assay. The livers of raccoons were collected within 3 h after their deaths from bodies that had been preserved on ice. The livers were immediately frozen in liquid nitrogen and preserved at −80 °C until the assay. All procedures for animal care and experiments were performed per the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Approval No. 18-0001). The animal experiments at the Graduate School of Hokkaido University were accredited by the Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International.

Extraction of NNIs from urine samples

The NNI compounds, including acetamiprid, N-desmethyl-acetamiprid (dm-acetamiprid), imidacloprid, clothianidin, dinotefuran, thiacloprid, thiamethoxam, and nitenpyram, were extracted and purified using a combination of quick, easy, cheap, effective, rugged, and safe (QuEChERS) and cold-induced aggregation microextraction methods (Song et al., 2019; Vichapong et al., 2016). First, 2 ml of urine sample was mixed with 2 ml of 16% sodium acetate buffer (0.5 g/3 ml double-distilled water) and 4 ml of 1% formic acid in acetonitrile. Subsequently, 50 μl of 100 ng/ml NNI internal standards containing deuterated labeled standards for acetamiprid (acetamiprid-d3), dm-acetamiprid-(dm-acetamiprid-d3), imidacloprid (imidacloprid-d4), clothianidin (clothianidin-d3), thiacloprid (thiacloprid-d4), thiamethoxam (thiamethoxam-d4), nitenpyram (nitenpyram-d3), and dinotefuran (dinotefuran-d3) were mixed by vortexing for 5 min. The mixtures were centrifuged at 10,000 *g* for 5 min and frozen overnight at −20 °C. The supernatants (400 μl) were filtered using Centricut W-MO (Kurabo Industries) by centrifuging at 12,000 *g* for 5 min. The refined samples were subjected to a solid phase extraction method using an ISOLUTE® HYDRO DME$^+$ 400 mg/3 ml cartridge (Biotage; Suwannarin et al., 2020) and eluted with 600 μl of acetonitrile. After dry-solidifying using a centrifugal evaporator (CVE-200D with UT-2000; EYELA), extracts were redissolved in 200 μl of 20% (v/v) methanol and transferred into vials for instrumental analysis.

Urine extract was analyzed using a liquid chromatography–mass spectrometer (LC–MS; 6495 Triple Quadrupole; Agilent Technologies) equipped with a Kinetex Biphenyl column (3.0 × 100 mm, φ2.7 μm; Phenomenex). Electrospray ionization (ESI) was applied. The solvent, gradient, oven temperature, and flow rate used for the liquid chromatography are shown in the Supporting Information, Table S2. Multiple-reaction monitoring was used for mass spectrometry, as shown in Table 1. The precision of the analysis for all eight NNIs was confirmed by multiple analyses, as shown in Table 1. The analysis was performed using the internal standards methods. Information regarding the internal standards is provided in the Supporting Information, Table S3. For each NNI compound, calibration curves were used in final concentrations of 0.01, 0.05, 0.1, 0.25, 0.5, 1, 2.5, 5, 10, 25, and 50 ng/ml. An NNI standard was added to canine urines that did not contain any NNIs, and extraction and purification were performed at each calibration point using the method just described. We confirmed that linearity (r^2) exceeded 0.99 for all the calibration curves. Procedural blank samples were prepared with each batch of 20 samples. No NNI compounds were detected from any procedural blank samples in our study. The method detection limit (MDL) and accuracy (percentage of recovery) were determined by replicate analyses ($n = 5$) of NNI-mixed canine urine that did not contain any NNIs and NNI-mixed double-distilled water at three different concentrations (1, 5, and 10 ng/ml). The practical quantitative limit (PQL) was settled as five times the MDL. The recovery rate and PQL are shown in Table 1. Urine samples from healthy dogs in which no NNI peak was detected were used for quality control. A standard mixture of NNI compounds was added to yield a urinary concentration of 5 μg/L. The quality control samples were injected with each batch of 20 samples. The between-day accuracy was within 80%–120%.

Measurement of urinary creatinine concentration

Urinary concentrations of NNIs were adjusted for creatinine to compensate for the hydration condition of raccoons between spot samples (Mage et al., 2008; Nimako et al., 2021). Urinary concentrations could be affected by the amount of water the raccoons drank before and after capture. Creatinine concentrations in urine were measured using the LC–ESI–MS/MS technique. Thawed urine samples were diluted 100 times in distilled water; thereafter, 10 μl of the diluted sample was supplemented with 990 μl of creatinine-d3 in acetonitrile. The sample was finally transferred into a high-performance liquid chromatography vial

TABLE 1: Selected neonicotinoids and their metabolites detected in raccoon urine samples

Target neonicotinoid	PQL (μg/L)	Recovery rate (%)	RSD (%)	MRM (*m/z*)	CE	Polarity for ESI
Acetamiprid	0.046	103	5	223.1 > 126.0	24	+
N-dm-acetamiprid	0.047	108	4	209.1 > 125.8	20	+
Imidacloprid	0.056	105	9	256.1 > 209.2	20	+
Clothianidin	0.064	106	10	250.0 > 132.0	16	+
Dinotefuran	0.29	96	3	203.1 > 157.0	12	+
Thiacloprid	0.027	99	7	253.0 > 126.0	28	+
Thiamethoxam	0.064	108	8	292.0 > 181.0	24	+
Nitenpyram	0.212	98	6	271.1 > 126.0	36	+

CE = collision energy; ESI = electrospray ionization; MRM = multiple-reaction monitoring; PQL = practical detection limit; RSD = relative standard deviation.

for analysis using LC–ESI–MS/MS (20 A series with LCMS8040; Shimadzu) equipped with a Shodex Asahipak NH2P-40 2D (2 × 150 mm ID) column (Showa Denko America). The LC–ESI–MS analysis was carried out in an isocratic mode. Details of the LC–ESI–MS/MS analysis, including solvent, gradient, oven temperature, flow rate, and selected *m/z* ions, are shown in the Supporting Information, Table S4. Urinary creatinine concentrations were calculated using the internal standards method, and the calibration curves were generated using seven-point calibration standards ranging from 1 to 500 ng/ml.

Preparation of hepatic microsomes for in vitro NNI metabolism assay

Liver samples of adult raccoons ($n = 3$) and 7-week-old Sprague–Dawley rats ($n = 4$) were used to obtain hepatic microsomal fractions. All animals used in this assay were male. Raccoons were caught in Hokkaido as described in the previous section, *Animals and treatment*, and no NNIs were detected in these raccoons. The Sprague–Dawley rats were purchased from Japan SLC. Hepatic microsomal fractions were prepared from raccoons and rats using the method described by Omura and Sato (1964) with slight modifications. The liver was homogenized with approximately three times the volume of 0.1 M potassium phosphate buffer (KPB; pH 7.4) and centrifuged at 9000 *g* at 4 °C for 20 min. The supernatants were filtered using gauze and centrifuged twice at 105,000 *g* and 4 °C for 60 min. Microsomal pellets at the bottom of centrifuge tubes were resuspended in 0.1 M KPB and preserved at −80 °C until subsequent use. The microsomal protein concentration was measured using the BCA protein kit (Thermo Fisher Scientific). The CYP concentration was determined using a reported method (Omura & Sato, 1964). The difference in the CO absorbance between 400 and 500 nm was examined by spectrophotometry (UV-2600; Shimadzu). The CYP concentration was calculated using the absorbance values at 450 and 490 nm.

In vitro CYP-dependent metabolism of NNIs

Metabolic assay of NNIs using hepatic microsomes was performed using a slight modification of the method described previously (Khidkhan et al., 2021): the protein concentrations of liver microsome were changed from 5 to 3 mg/ml for both raccoons and rats. The incubation mixture consisted of KPB (0.1 M, pH 7.4), MgCl$_2$ (3 mM in final concentration), glucose-6-phosphate (5 mM), and hepatic microsomes (3 mg/ml of protein concentrations). As a substrate, imidacloprid (Kanto Chemical), clothianidin (Wako Pure Chemical), acetamiprid (Cosmo Bio), or thiamethoxam (Dr. Ehrenstorfer) in 3% methanol were added at final concentrations of 10, 25, 50, 100, 200, and 400 µM. After preincubation at 37 °C for 5 min, the enzymatic reaction was initiated by adding glucose-6-phosphate dehydrogenase (2 IU/ml) and nicotinamide adenine dinucleotide phosphate hydrogen (0.5 mM). After incubation for 30 min, 200 µM of 1% formic acid in acetonitrile was mixed to terminate the enzymatic reaction. Samples were then centrifuged at 15,000 *g* for 10 min,

and the supernatant was filtered using a GL Chromato Disk sample filter (pore size, 0.2 µM; GL Sciences). We performed all assays in duplicate for each concentration. Negative control mixtures without substrate were used for each sample.

An LC–ESI–MS/MS device (6495 Triple Quadruple; Agilent Technologies) equipped with a Kinetex Biphenyl column (3.0 × 100 mm, φ2.7 µm; Phenomenex) was used to measure the target metabolites of imidacloprid, clothianidin, acetamiprid, and thiamethoxam. In the previous study (Khidkhan et al., 2021), the following metabolites were produced by CYP-dependent in vitro metabolic assay in several mammalian species: 4OH-imidacloprid, 5OH-imidacloprid, desmethyl-clothianidin, and desmethyl-acetamiprid. Therefore, the same metabolites were measured in our study; 4OH-imidacloprid, 5OH-imidacloprid and desmethyl-clothianidin were synthesized at Toho University, Chiba, Japan, and desmethyl-acetamiprid was purchased from Sigma-Aldrich. Details of the LC–EIS–MS/MS analysis, including solvent, gradient, oven temperature, flow rate, collision energy, and selected ions are shown in the Supporting Information, Table S5.

Calculation of estimated daily intake of NNIs from urinary concentrations

Estimated daily intake amounts of NNIs in raccoons were calculated from creatinine-adjusted NNI concentrations using the following formula:

Estimated daily intake amount/body weight (µg/kg/day) = urinary NNI concentration (µg/g creatinine) × maximum daily creatinine excretion amount/body weight in dog (0.04 g/kg/day) × 1/*r* (urinary excretion correction coefficient).

The following values were applied for the urinary excretion correction coefficients: $r = 0.586$ for acetamiprid, $r = 0.133$ for imidacloprid, $r = 0.596$ for clothianidin, $r = 0.899$ for dinotefuran, $r = 0.05$ for thiacloprid, $r = 0.6$ for thiamethoxam (Ikenaka et al., 2019). These *r* values were obtained from previous studies on humans.

Statistical analysis

Statistical analyses were conducted using the JMP® Pro 16 software (SAS Institute). For the calculations of ∑NNIs, concentrations below PQL were set to zero. For the statistical significance analysis, concentrations of NNIs below the PQL were set as the PQL value divided by the square root of 2 (Hornung & Reed, 1990). The Michaelis–Menten equation was used to calculate kinetic parameters such as maximum velocity (V_{max}), Michaelis–Menten constant (K_m), and V_{max}/K_m ratio in GraphPad Prism Ver 8.0 for Windows (GraphPad Prism Software). Student's *t*-test was performed to compare the V_{max}/K_m ratio between raccoons and rats. Mann–Whitney's *U* test was applied for the comparison of creatinine-adjusted NNI concentration. Creatinine-adjusted NNI concentrations between sex and age were compared using Mann–Whitney's *U* test. The Steel Dwass test was used for comparison of creatinine-adjusted NNI concentration among sampling months between April and October.

A *p*-value < 0.05 was considered statistically significant for all analyses.

RESULTS AND DISCUSSION

Detection of NNIs in the urine of raccoons

The urinary concentrations of NNI compounds in raccoon samples were measured and corrected using creatinine concentrations in urine. The volume-based and creatinine-adjusted NNI concentrations are shown in Tables 2 and 3, respectively. Six parent NNI compounds (acetamiprid, imidacloprid, clothianidin, dinotefuran, thiacloprid, and thiamethoxam) and one NNI metabolite (dm-acetamiprid) were detected at the following frequencies: clothianidin (66.1%) > dm-acetamiprid (52.5%) > acetamiprid (35.6%) > imidacloprid (23.7%) > dinotefuran (20.3%) > thiamethoxam (18.6%) > thiacloprid (3.4%). The compound nitenpyram was not detected in all the raccoons. Either one of the six NNIs or one metabolite was detected in samples from 90% of the raccoons (53/59). There was no significant difference between sex, age, and month of capture in all NNI compounds. Ours is the first study to report the detection of NNI compounds in wild raccoons. Previously, NNI residues were found in wild terrestrial vertebrates, including wild boar, deer, and birds (Byholm et al., 2018; ńKaczyński et al., 2021; MacDonald et al., 2018). Although NNIs were extracted from the blood and organs of wildlife species in these previous studies, we used urine samples from raccoons, and could therefore detect NNIs more frequently. The frequent detection of these NNI compounds in urine samples suggests that NNIs could be omnipresent among the population of wild raccoons in Hokkaido. In Japan, NNIs are often applied to agricultural crops, including corn and fruits, which are often eaten by raccoons (Osaki et al., 2019; Taira, 2014). It is possible that residues of NNIs applied to agricultural fields in the capture areas of our study might have transferred through crops into wild raccoons. We found the remains of crops in the stomachs of more than one-fourth of the raccoons.

Urinary concentrations of NNIs in the raccoons determined in the present study were compared with those in Japanese people reported in a previous study (Ikenaka et al., 2019; Table 4). Creatinine-adjusted concentrations were used for comparison to correct for the hydration conditions of raccoons among spot samples, because raccoons might have consumed different amounts of water before and after capture, and their urine might be concentrated. Creatinine-based correction between different animal species is now commonly used to compensate for urinary levels of various compounds, including hormones and herbicides (Karthikraj & Kannan, 2019; Syme et al., 2007). There are no data on raccoons; the daily excreted amounts of creatinine in the urine are almost the same between dogs and humans (dogs: ~20–40 mg/kg/day; humans: ~1000–2000 mg/day in adults [20–40 mg/kg/day for a body weight of 50 kg]; Bingham et al., 1988; Braun et al., 2003). In the taxonomy of mammals, raccoons and dogs are classified as Carnivora; thus dogs are much closer to raccoons than humans. Therefore, we assumed that it is possible to evaluate the exposure levels of raccoons by comparing the creatinine-adjusted urinary concentrations of NNIs with those of humans. Overall, the detected concentrations of NNIs were not significantly different between raccoons and humans (Table 4). However, the results of acetamiprid and nitenpyram were different. Raccoons showed a significantly higher acetamiprid concentration than humans (mean value; raccoons: 0.28 µg/g creatinine; humans: 0.043 µg/g creatinine). In Japan, acetamiprid is often applied to crops, such as vegetables and fruits (Fujita et al., 2012). In addition, nitenpyram was not detected in the raccoons in our study even though it has been detected in humans at a rate of 30% (Ikenaka et al., 2019). The shipment amount of nitenpyram in Hokkaido was the lowest of the seven NNI parent compounds in 2020, at only 5 tons/year, which is approximately 1.2% of the total shipment amount for the seven NNIs (National Institute for Environmental Studies, 2022). Therefore, nitenpyram might not have been applied to crops at the sampling site in our study.

Most of the exposed acetamiprid is metabolized to dm-acetamiprid and excreted through urine in mice (Ford & Casida, 2006). The metabolite dm-acetamiprid was more frequently and abundantly detected than acetamiprid in raccoons, which suggests that it can be used as a marker for acetamiprid exposure in wild raccoons as it is in humans (Harada et al., 2016; Marfo et al., 2015). In the future, it might be possible to detect NNIs with higher sensitivity by measuring various metabolites simultaneously. However, it has been

TABLE 2: Statistical summary of volume-adjusted concentrations of neonicotinoids in the urine samples of raccoons (*n* = 59)

| Neonicotinoid | >PQL (%)[a] | >PQL of DIN (%)[b] | GM ± SD (µg/L) | Percentile (µg/L) | | | | | PQL |
				25th	50th	75th	95th	100th	
Acetamiprid	35.59	8.47	0.07 ± 0.20	<PQL	<PQL	0.23	0.33	1.29	0.046
N-dm-Acetamiprid	52.54	20.34	0.11 ± 9.33	<PQL	<PQL	0.22	3.28	71.28	0.047
Imidacloprid	23.73	8.47	0.06 ± 0.20	<PQL	<PQL	<PQL	0.36	1.45	0.056
Clothianidin	66.10	32.20	0.17 ± 0.89	<PQL	0.14	0.45	2.62	4.33	0.064
Dinotefuran	20.34	20.34	0.25 ± 0.76	<PQL	<PQL	<PQL	1.11	5.67	0.29
Thiacloprid	3.39	1.69	0.02 ± 0.04	<PQL	<PQL	<PQL	<PQL	0.32	0.027
Thiamethoxam	18.64	6.78	0.07 ± 0.95	<PQL	<PQL	<PQL	0.42	7.30	0.064
Nitenpyram	0	0	—	<PQL	<PQL	<PQL	<PQL	<PQL	0.212
∑8NNIs	—	—	3.11 ± 10.15	0.52	0.79	1.79	12.16	76.93	—

[a]Percentage detection frequency.
[b]Percentage detection frequency above the PQL of dinotefuran (0.29 µg/L).
DIN = dinotefuran; GM = geometric mean; PQL = practical detection limit; ∑8NNIs = sum of creatinine-adjusted concentrations of neonicotinoid compounds.

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TABLE 3: Statistical summary of creatinine-adjusted concentrations of neonicotinoids in the urine samples of raccoons ($n = 59$)

Neonicotinoid	>PQL (%)[a]	> PQL of DIN (%)[b]	GM ± SD (µg/g creatinine)	Percentile (µg/g creatinine) 25th	50th	75th	95th	100th	PQL
Acetamiprid	35.59	8.47	0.10 ± 0.74	<PQL	<PQL	0.25	0.79	5.61	0.053
N-dm-acetamiprid	52.54	20.34	0.16 ± 10.78	<PQL	<PQL	0.35	8.28	76.56	0.054
Imidacloprid	23.73	8.47	0.08 ± 0.75	<PQL	<PQL	<PQL	0.43	5.76	0.064
Clothianidin	66.1	32.20	0.26 ± 1.65	<PQL	0.21	0.43	4	9.4	0.074
Dinotefuran	20.34	20.34	0.37 ± 1.64	<PQL	<PQL	<PQL	1.38	10.28	0.33
Thiacloprid	3.39	1.69	0.03 ± 0.44	<PQL	<PQL	<PQL	<PQL	3.41	0.031
Thiamethoxam	18.64	6.78	0.10 ± 0.95	<PQL	<PQL	<PQL	0.5	6.96	0.074
Nitenpyram	0	0	—	<PQL	<PQL	<PQL	<PQL	<PQL	0.24
∑8NNIs	—	—	1.60 ± 13.13	0.79	1.36	2.53	15.98	82.62	—

[a]Percentage detection frequency.
[b]Percentage detection frequency above the PQL of dinotefuran (0.29 µg/g creatinine).
DIN = dinotefuran; GM = geometric mean; PQL = practical detection limit; ∑8NNIs = sum of creatinine-adjusted concentrations of neonicotinoid compounds.

confirmed that acetamiprid in environmental soils is easily converted to dm-acetamiprid by soil microorganisms (Rambhade et al., 2010). Therefore, direct exposure of dm-acetamiprid to raccoons via environmental materials such as soil and water in the present study is also possible.

It should be noted that detected frequencies are not strictly comparable among each NNI compound because the PQLs are different (e.g., 0.03 µg/L for thiacloprid and 0.29 µg/L for dinotefuran). The detected frequency will tend to be higher as the PQL becomes lower. For strict comparison among NNI compounds, detected rates above the PQL of dinotefuran (0.29 µg/L and 0.33 µg/g creatinine), which had the highest PQL value, were calculated as shown in Tables 2 and 3. The detected frequencies were as follows: clothianidin (32.2%) > dm-acetamiprid (20.3%) = dinotefuran (20.3%) > acetamiprid (8.5%) = imidacloprid (8.5%) > thiamethoxam (6.8%) > thiacloprid (1.7%). Apart from dinotefuran, the order of the detected rates did not different from when the PQLs of each NNI were used. When the PQL of dinotefuran was used, the detected order of dinotefuran was higher than when the PQLs of each NNI were used. This indicates that it is important to apply a common PQL value when detected rates among multiple NNIs are compared.

CYP-dependent metabolic ability of NNIs in the liver

An in vitro acetamiprid metabolism assay confirmed the formation of dm-acetamiprid in both raccoons and rats. As shown in Figure 1A and Table 5, the V_{max}/K_m values for

dm-acetamiprid formation were significantly lower in raccoons than in rats ($p = 0.008$). In the metabolic assay using imidacloprid, 4OH-imidacloprid and 5OH-imidacloprid were produced in both raccoons and rats. Significantly lower V_{max}/K_m values for 4OH-imidacloprid and 5-OH imidacloprid formation were found in raccoons than in rats ($p = 0.046$ for 4OH-imidacloprid; $p = 0.036$ for 5OH-imidacloprid; Figure 1C and D and Table 5). In the assay for clothianidin, it was revealed that dm-clothianidin was produced in both raccoons and rats. When mice are exposed to clothianidin, dm-clothianidin is the most abundantly detected metabolite in the brain and liver (Ford & Casida, 2006). As shown in Figure 1B and Table 5, the ability of raccoon microsomes to metabolize clothianidin to dm-clothianidin was lower than that of rat microsomes ($p = 0.031$). In addition, it was found that the ability to convert thiamethoxam to clothianidin in raccoons was much lower than in rats ($p = 0.001$), as shown in Figure 1E and Table 5.

Among all the NNI metabolites we analyzed, raccoon microsomes showed much lower metabolic capacities than those of rats; V_{max}/K_m values in raccoons were approximately one-tenth to one-third those in rats. Metabolism of NNIs is efficiently performed by CYP, and CYP-dependent metabolism is one of the most important factors in determining the chemosensitivity of NNIs (Simon-Delso et al., 2015; Thompson et al., 2020). Based on our results, the difference in the metabolic abilities of NNIs between raccoons and rats could be dependent on the differences in expression levels and functions of each CYP isoform. It has been reported that NNIs such as clothianidin and thiamethoxam are metabolized by CYP

TABLE 4: Urinary neonicotinoid concentrations (µg/g creatinine) in raccoons compared with that in Japanese people

Subject	Measure	ACE	dm-ACP	IMI	CLO	DIT	TCP	TMXM	NIT
Raccoons: present study ($n = 59$)	DF (%)	35.59	52.54	23.73	66.1	20.34	3.39	18.64	0
	Mean	0.28	2.48	0.23	0.81	0.74	0.09	0.29	0
	Maximum	5.61	76.56	5.76	9.4	10.28	3.41	6.96	0
Japanese people: Ikenaka et al. (2019; $n = 45$)	DF (%)	11.11	86.67	17.78	48.89	48.89	33.33	33.33	26.67
	Mean	0.043	2.2	0.13	1.06	0.57	0.03	0.17	0.1
	Maximum	1.03	26.92	1.59	15.06	9.49	0.2	2.11	1.25

ACE = acetamiprid; CLO = clothianidin; DIT = dinotefuran; DF = detection frequency; dm-ACP = dimethyl acetamiprid; IMI = imidacloprid; NIT = nitenpyram; TCP = thiacloprid; TMXM = thiamethoxam.
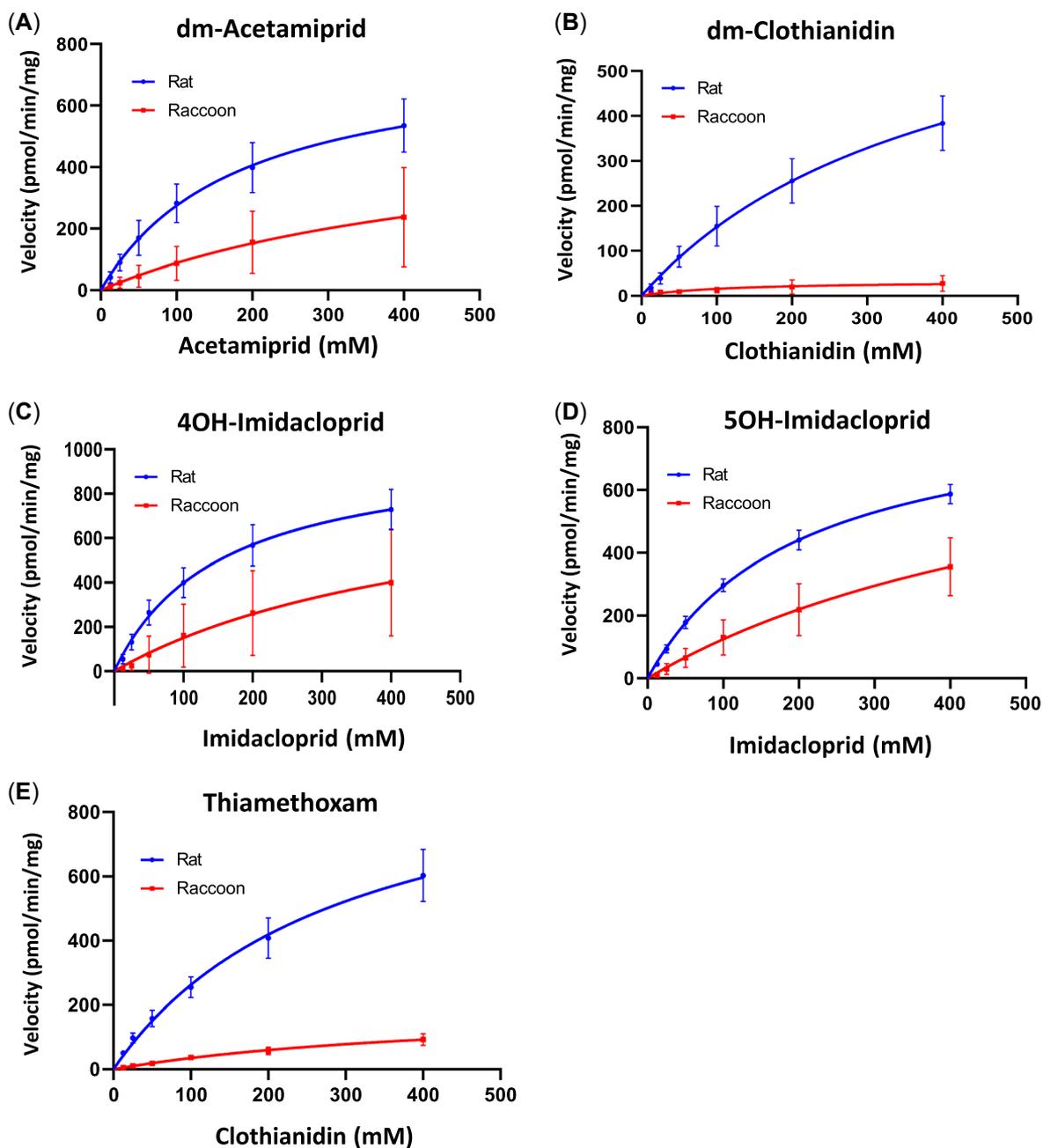
FIGURE 1: (A–E) Michaelis–Menten plots for cytochrome P450 (CYP) activity of neonicotinoid insecticides in Sprague–Dawley (SD) rat (blue circles) and raccoon (red squares) liver microsomes. Each value is shown as mean ± SD.

2A6, 2B6, 2C19, and 3A4 in humans (Shi et al., 2009). Although there are no data on CYP isoforms that metabolize acetamiprid, the metabolic abilities of these CYP isoforms may be lower in raccoons than in rats. The results of a metabolic assay using liver microsomes indicate that raccoons might be more susceptible to NNIs than rats. Further studies should be performed to identify interspecies differences in NNI metabolism in the livers of several other wild mammalian species.

Estimated risk of NNIs in wild raccoons

The urinary concentrations of NNIs in raccoons were on the same level as those in humans, suggesting that raccoons could be exposed to comparable levels of NNIs in the environment. Raccoons live near farmlands, eat agricultural crops in Hokkaido (Osaki et al., 2019), and are therefore frequently exposed to multiple NNIs. Although the exact risks associated with such multiple exposures in mammals are not well understood, additive and synergistic toxicities associated with multiple exposures can be of concern. Emerging reports suggest that NNI exposure can have neurotoxic effects on mammals even at low exposure doses. For example, in mice, anxiety-related behavior was induced by exposure to a considerably low dose of clothianidin, less than the no-observable-adverse-effect levels (NOAEL; ~50 mg/kg in a 78-week carcinogenicity study; Hirano et al., 2018). As shown in Table 6, daily intake

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TABLE 5: Michaelis–Menten kinetics parameters (V_{max} [pmol/min/mg], K_m [µM], V_{max}/K_m [µL/min/mg]; mean $\pm$ SD) for cytochrome P450 (CYP) metabolism of neonicotinoids

Substrate	Metabolite	Parameter	Rat	Raccoon	Significance
Acetamiprid	dm-Acetamiprid	V_{max}/K_m	4.3 ± 1.3	1.1 ± 0.6	—*
		V_{max}	795 ± 75	769 ± 365	
		K_m	203 ± 62	1109 ± 981	
Imidacloprid	4OH-imidacloprid	V_{max}/K_m	6.6 ± 1.5	1.9 ± 1.3	—*
		V_{max}	1013 ± 66	2820 ± 1764	
		K_m	159 ± 25	657 ± 369	
	5OH-imidacloprid	V_{max}/K_m	4.4 ± 0.4	1.5 ± 0.5	—*
		V_{max}	882 ± 19	1143 ± 256	
		K_m	201 ± 17	985 ± 544	
Clothianidin	dm-Clothianidin	V_{max}/K_m	2.0 ± 0.6	0.7 ± 0.5	—*
		V_{max}	866 ± 201	36 ± 26	
		K_m	506 ± 214	103 ± 80	
Thiamethoxam	Clothianidin	V_{max}/K_m	3.5 ± 0.6	0.4 ± 0.0	—*
		V_{max}	1055 ± 161	203 ± 43	
		K_m	305 ± 52	467 ± 64	

*Statistically significant differences in V_{max}/K_m between rat and raccoon (Student's t-test, $p < 0.05$).

TABLE 6: Estimated daily intake amounts of neonicotinoid insecticides from urinary concentrations (µg/kg/day) in raccoons ($n = 59$)

Neonicotinoid	GM $\pm$ SD (µg/kg/day)	Percentile (µg/kg/day)				
		25th	50th	75th	95th	100th
Acetamiprid	0.019 ± 0.051	<PQL	<PQL	0.017	0.054	0.383
Imidacloprid	0.069 ± 0.225	<PQL	<PQL	<PQL	0.129	1.731
Clothianidin	0.054 ± 0.111	<PQL	0.014	0.029	0.268	0.631
Dinotefuran	0.033 ± 0.073	<PQL	<PQL	<PQL	0.061	0.457
Thiacloprid	0.075 ± 0.349	<PQL	<PQL	<PQL	<PQL	2.729
Thiamethoxam	0.019 ± 0.063	<PQL	<PQL	<PQL	0.033	0.464
Σ6NNIs	0.269 ± 0.713	0.065	0.113	0.213	0.630	5.513

GM = geometric mean; PQL = practical detection limit; Σ6NNIs = sum of estimated daily intake amounts of neonicotinoid compounds.

amounts of NNIs were estimated from urinary concentrations. The maximum estimated cumulative intake amount for the six NNIs was 5.51 µg/kg/day. Based on this estimation, exposure levels of NNIs in this raccoon population were much lower than NOAEL levels.

Because the enzymatic activities of CYP, the major metabolic pathway for NNIs, were lower in raccoons, they might be more susceptible than humans or experimental animals. The typical toxic effects of low-dose NNI exposure are based on neurodisruption, including increased frequency of anxiety-like behavior and decreased memory capacities in experimental animals (Cimino et al., 2017; Hirano et al., 2015). Therefore, it is possible that the behavior of raccoons might be somewhat affected by frequent NNI exposure, although the amounts detected in wild raccoons in the present study were far lower than the exposure dose in previous animal experiments; generally, approximately 5–50 mg/kg of exposure is used in mice and rats for the assessment of NNI-induced neurotoxicities (Sheets et al., 2016). In wildlife species, nonlethal toxic effects that have only slight changes at individual levels can have a significant impact at the population and ecosystem levels (Hay, 2009; Köhler & Triebskorn, 2013). The NNIs are believed to have high selective toxicity in insects and lower toxic effects in mammals (Simon-Delso et al., 2015; Wang et al., 2018), but there are concerns about unknown toxicity in wildlife species;

thus, biomonitoring and research, taking interspecies differences into account, will be needed in the future.

CONCLUSIONS

In the present study, six parent NNI compounds and one NNI metabolite were detected in wild raccoons from Hokkaido. Either one of the six NNIs or one metabolite were detected in 90% of raccoons (53/59), indicating that most raccoons were exposed to NNIs in the environment. Most of the detected NNIs in raccoons were not significantly different from the urinary concentrations reported previously in humans. The results indicate that raccoons can be frequently exposed to NNIs in agricultural areas. In addition, in vitro metabolic assays suggested that raccoons could have a low ability to metabolize NNIs in the liver: the V_{max}/K_m values for raccoons were one-tenth to one-third those for rats. Our results will contribute to a better understanding of the estimation and evaluation of NNI-induced toxic effects on raccoons and could finally result in the enhanced conservation of wild mammalian species.

Conflict of Interest—The authors declare no conflicts of interest.

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REFERENCES

Bingham, S. A., Williams, R., Cole, T. J., Price, C. P., & Cummings, J. H. (1988). Reference values for analytes of 24-h urine collections known to be complete. *Annals of Clinical Biochemistry*, 25(6), 610–619. https://doi.org/10.1177/000456328802500603

Blus, L. J. (2011). DDT, DDD, and DDE in birds. In W. N. Bey & J. P. Meador (Eds.), *Environmental contaminants in biota* (2nd ed., pp. 425–446). CRC Press. https://doi.org/10.1201/b10598-13

Braun, J. P., Lefebvre, H. P., & Watson, A. D. J. (2003). Creatinine in the dog: A review. *Veterinary Clinical Pathology*, 32(4), 162–179. https://doi.org/10.1111/j.1939-165X.2003.tb00332.x

Byholm, P., Mäkeläinen, S., Santangeli, A., & Goulson, D. (2018). First evidence of neonicotinoid residues in a long-distance migratory raptor, the European honey buzzard (*Pernis apivorus*). *Science of the Total Environment*, 639, 929–933. https://doi.org/10.1016/j.scitotenv.2018.05.185

Casida, J. E. (2018). Neonicotinoids and other insect nicotinic receptor competitive modulators: Progress and prospects. *Annual Review of Entomology*, 63, 125–144. https://doi.org/10.1146/annurev-ento-020117-043042

Cimino, A. M., Boyles, A. L., Thayer, K. A., & Perry, M. J. (2017). Effects of neonicotinoid pesticide exposure on human health: A systematic review. *Environmental Health Perspectives*, 125(2), 155–162. https://doi.org/10.1289/EHP515

Eng, M. L., Stutchbury, B. J. M., & Morrissey, C. A. (2019). A neonicotinoid insecticide reduces fueling and delays migration in songbirds. *Science*, 365(6458), 1177–1180. https://doi.org/10.1126/science.aaw9419

Ford, K. A., & Casida, J. E. (2006). Unique and common metabolites of thiamethoxam, clothianidin, and dinotefuran in mice. *Chemical Research in Toxicology*, 19(11), 1549–1556. https://doi.org/10.1021/tx0601859

Fujita, M., Yajima, T., Iijima, K., & Sato, K. (2012). Comparison of the variability in the levels of pesticide residue observed in Japanese cabbage and grape units. *Journal of Agricultural and Food Chemistry*, 60(6), 1516–1521. https://doi.org/10.1021/jf2040059

Gibbons, D., Morrissey, C., & Mineau, P. (2015). A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. *Environmental Science and Pollution Research International*, 22(1), 103–118. https://doi.org/10.1007/s11356-014-3180-5

Grue, C. E., Gibert, P. L., & Seeley, M. E. (1997). Neurophysiological and behavioral changes in non-target wildlife exposed to organophosphate and carbamate pesticides: Thermoregulation, food consumption, and reproduction. *American Zoologist*, 37(4), 369–388. https://doi.org/10.1093/icb/37.4.369

Harada, K. H., Tanaka, K., Sakamoto, H., Imanaka, M., Niisoe, T., Hitomi, T., Kobayashi, H., Okuda, H., Inoue, S., Kusakawa, K., Oshima, M., Watanabe, K., Yasojima, M., Takasuga, T., & Koizumi, A. (2016). Biological monitoring of human exposure to neonicotinoids using urine samples, and neonicotinoid excretion kinetics. *PLoS One*, 11(1), e0146335. https://doi.org/10.1371/journal.pone.0146335

Hay, M. E. (2009). Marine chemical ecology: Chemical signals and cues structure marine populations, communities, and ecosystems. *Annual Review of Marine Science*, 1, 193–212. https://doi.org/10.1146/annurev.marine.010908.163708

Herceg Romanić, S. H., Klinčić, D., Kljakovic-Gašpić, Z., Kusak, J., Reljić, S., & Huber, D. (2015). Organochlorine pesticides and polychlorinated biphenyl congeners in wild terrestrial mammals from Croatia: Interspecies comparison of residue levels and compositions. *Chemosphere*, 137(15), 52–58. https://doi.org/10.1016/j.chemosphere.2015.05.026

Hirano, T., Yanai, S., Omotehara, T., Hashimoto, R., Umemura, Y., Kubota, N., Minami, K., Nagahara, D., Matsuo, E., Aihara, Y., Shinohara, R., Furuyashiki, T., Mantani, Y., Yokoyama, T., Kitagawa, H., & Hoshi, N. (2015). The combined effect of clothianidin and environmental stress on the behavioral and reproductive function in male mice. *Journal of Veterinary Medical Science*, 77(10), 1207–1215. https://doi.org/10.1292/jvms.15-0188

Hirano, T., Yanai, S., Takada, T., Yoneda, N., Omotehara, T., Kubota, N., Minami, K., Yamamoto, A., Mantani, Y., Yokoyama, T., Kitagawa, H., & Hoshi, N. (2018). NOAEL-dose of a neonicotinoid pesticide, clothianidin, acutely induce anxiety-related behavior with human-audible vocalizations in male mice in a novel environment. *Toxicology Letters*, 282, 57–63. https://doi.org/10.1016/j.toxlet.2017.10.010

Hornung, R. W., & Reed, L. D. (1990). Estimation of average concentration in the presence of nondetectable values. *Applied Occupational and Environmental Hygiene*, 5(1), 46–51. https://doi.org/10.1080/1047322X.1990.10389587

Ikeda, T., Asano, M., Matoba, Y., & Abe, G. (2004). Present status of invasive alien raccoon and its impact in Japan. *Global Environmental Research*, 8(2), 125–131.

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Ikenaka, Y., Miyabara, Y., Ichise, T., Nakayama, S., Nimako, C., Ishizuka, M., & Tohyama, C. (2019). Exposures of children to neonicotinoids in pine wilt disease control areas. *Environmental Toxicology and Chemistry*, *38*(1), 71–79. https://doi.org/10.1002/etc.4316

Ishizuka, M., Lee, J. J., Masuda, M., Akahori, F., Kazusaka, A., & Fujita, S. (2006). CYP2D-related metabolism in animals of the Canoidea superfamily—Species differences. *Veterinary Research Communications*, *30*(5), 505–512. https://doi.org/10.1007/s11259-006-3213-8

Junge, R. E., & Hoffmeister, D. F. (1980). Age determination in raccoons from cranial suture obliteration. *Journal of Wildlife Management*, *44*(3), 725–729. https://doi.org/10.2307/3808029

Kaczyński, P., Łozowicka, B., Perkowski, M., Zoń, W., Hrynko, I., Rutkowska, E., & Skibko, Z. (2021). Impact of broad-spectrum pesticides used in the agricultural and forestry sector on the pesticide profile in wild boar, roe deer and deer and risk assessment for venison consumers. *Science of the Total Environment*, *784*, 147215. https://doi.org/10.1016/j.scitotenv.2021.147215

Karthikraj, R., & Kannan, K. (2019). Widespread occurrence of glyphosate in urine from pet dogs and cats in New York State, USA. *Science of the Total Environment*, *659*, 790–795. https://doi.org/10.1016/j.scitotenv.2018.12.454

Khidkhan, K., Ikenaka, Y., Ichise, T., Nakayama, S. M. M., Mizukawa, H., Nomiyama, K., Iwata, H., Arizono, K., Takahashi, K., Kato, K., & Ishizuka, M. (2021). Interspecies differences in cytochrome P450-mediated metabolism of neonicotinoids among cats, dogs, rats, and humans. *Comparative Biochemistry and Physiology: Toxicology and Pharmacology*, *239*, 108898. https://doi.org/10.1016/j.cbpc.2020.108898

Köhler, H. R., & Triebskorn, R. (2013). Wildlife ecotoxicology of pesticides: Can we track effects to the population level and beyond? *Science*, *341*(6147), 759–765. https://doi.org/10.1126/science.1237591

Kwon, Y. K., Wee, S. H., & Kim, J. H. (2004). Pesticide poisoning events in wild birds in Korea from 1998 to 2002. *Journal of Wildlife Diseases*, *40*(4), 737–740. https://doi.org/10.7589/0090-3558-40.4.737

Lundin, O., Rundlöf, M., Smith, H. G., Fries, I., & Bommarco, R. (2015). Neonicotinoid insecticides and their impacts on bees: A systematic review of research approaches and identification of knowledge gaps. *PLoS One*, *10*(8), e0136928. https://doi.org/10.1371/journal.pone.0136928

MacDonald, A. M., Jardine, C. M., Thomas, P. J., & Nemeth, N. M. (2018). Neonicotinoid detection in wild turkeys (*Meleagris gallopavo silvestris*) in Ontario, Canada. *Environmental Science and Pollution Research International*, *25*(16), 16254–16260. https://doi.org/10.1007/s11356-018-2093-0

Mage, D. T., Allen, R. H., & Kodali, A. (2008). Creatinine corrections for estimating children's and adult's pesticide intake doses in equilibrium with urinary pesticide and creatinine concentrations. *Journal of Exposure Science and Environmental Epidemiology*, *18*(4), 360–368. https://doi.org/10.1038/sj.jes.7500614

Marfo, J. T., Fujioka, K., Ikenaka, Y., Nakayama, S. M. M., Mizukawa, H., Aoyama, Y., Ishizuka, M., & Taira, K. (2015). Relationship between urinary n-desmethyl-acetamiprid and typical symptoms including neurological findings: A prevalence case-control study. *PLoS One*, *10*(11), e0142172. https://doi.org/10.1371/journal.pone.0142172

National Institute for Environmental Studies. (2022). Public database for shipping amount of insecticides in Japan. Retrieved January 6, 2022, from https://www.nies.go.jp/kisplus/src_dvr/dvr

Nimako, C., Ikenaka, Y., Akoto, O., Bortey-Sam, N., Ichise, T., Nakayama, S. M. M., Asante, K. A., Fujioka, K., Taira, K., & Ishizuka, M. (2021). Human exposures to neonicotinoids in Kumasi, Ghana. *Environmental Toxicology and Chemistry*, *40*(8), 2306–2318. https://doi.org/10.1002/etc.5065

Omura, T., & Sato, R. (1964). The carbon monoxide-binding pigment of liver microsomes. *Journal of Biological Chemistry*, *239*(7), 2370–2378. https://doi.org/10.1016/S0021-9258(20)82244-3

Osaki, A., Sashika, M., Abe, G., Shinjo, K., Fujimoto, A., Nakai, M., Shimozuru, M., & Tsubota, T. (2019). Comparison of feeding habits and habitat use between invasive raccoons and native raccoon dogs in Hokkaido, Japan. *BMC Ecology*, *19*(1), 1–15. https://doi.org/10.1186/s12898-019-0249-5

Pisa, L., Goulson, D., Yang, E. C., Gibbons, D., Sánchez-Bayo, F., Mitchell, E., Aebi, A., van der Sluijs, J., MacQuarrie, C. J. K., Giorio, C., Long, E. Y., McField, M., Bijleveld van Lexmond, M., & Bonmatin, J. M. (2021). An update of the Worldwide Integrated Assessment (WIA) on systemic insecticides. Part 2: Impacts on organisms and ecosystems. *Environmental Science and Pollution Research International*, *28*(10), 11749–11797. https://doi.org/10.1007/s11356-017-0341-3

Rambhade, S., Chakraborty, A., Patil, U., & Rambhade, A. (2010). Diabetes mellitus—Its complications, factors influencing complications and prevention—An overview. *Journal of Chemical and Pharmaceutical Research*, *2*(6), 7–25.

Roberts, J. R., Dawley, E. H., & Reigart, J. R. (2019). Children's low-level pesticide exposure and associations with autism and ADHD: A review. *Pediatric Research*, *85*(2), 234–241. https://doi.org/10.1038/s41390-018-0200-z

Sheets, L. P., Li, A. A., Minnema, D. J., Collier, R. H., Creek, M. R., & Peffer, R. C. (2016). A critical review of neonicotinoid insecticides for developmental neurotoxicity. *Critical Reviews in Toxicology*, *46*(2), 153–190. https://doi.org/10.3109/10408444.2015.1090948

Shi, X., Dick, R. A., Ford, K. A., & Casida, J. E. (2009). Enzymes and inhibitors in neonicotinoid insecticide metabolism. *Journal of Agricultural and Food Chemistry*, *57*(11), 4861–4866. https://doi.org/10.1021/jf900250f

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L. P., Bonmatin, J. M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D. W., Giorio, C., Girolami, V., Goulson, D., Kreutzweiser, D. P., Krupke, C. H., Liess, M., Long, E., Mcfield, M., Mineau, P., Mitchell, E. A., Morrissey, C. A., … Wiemers, M. (2015). Systemic insecticides (neonicotinoids and fipronil): Trends, uses, mode of action and metabolites. *Environmental Science and Pollution Research International*, *22*(1), 5–34. https://doi.org/10.1007/s11356-014-3470-y

Song, S., He, Y., Zhang, B., Gui, M., Ouyang, J., & Zhang, T. (2019). A novel extraction method for simultaneous determination of neonicotinoid insecticides and their metabolites in human urine. *Analytical Methods*, *11*(19), 2571–2578. https://doi.org/10.1039/C9AY00288J

Suwannarin, N., Prapamontol, T., Isobe, T., Nishihama, Y., & Nakayama, S. F. (2020). Characteristics of exposure of reproductive-age farmworkers in Chiang Mai Province, Thailand, to organophosphate and neonicotinoid insecticides: A pilot study. *International Journal of Environmental Research and Public Health*, *17*(21), 7871. https://doi.org/10.3390/ijerph17217871

Syme, H. M., Fletcher, M. G., Bailey, S. R., & Elliott, J. (2007). Measurement of aldosterone in feline, canine, and human urine. *Journal of Small Animal Practice*, *48*(4), 202–208. https://doi.org/10.1111/j.1748-5827.2006.00264.x

Taira, K. (2014). Human neonicotinoids exposure in Japan. *Japanese Journal of Clinical Ecology*, *23*(1), 14–24.

Thompson, D. A., Lehmler, H. J., Kolpin, D. W., Hladik, M. L., Vargo, J. D., Schilling, K. E., LeFevre, G. H., Peeples, T. L., Poch, M. C., LaDuca, L. E., Cwiertny, D. M., & Field, R. W. (2020). A critical review on the potential impacts of neonicotinoid insecticide use: Current knowledge of environmental fate, toxicity, and implications for human health. *Environmental Science: Processes and Impacts*, *22*(6), 1315–1346. https://doi.org/10.1039/C9EM00586B

Van Den Berg, H., Manuweera, G., & Konradsen, F. (2017). Global trends in the production and use of DDT for control of malaria and other vector-borne diseases. *Malaria journal*, *16*(1), 401. https://doi.org/10.1186/s12936-017-2050-2

Vichapong, J., Burakham, R., Santaladchaiyakit, Y., & Srijaranai, S. (2016). A preconcentration method for analysis of neonicotinoids in honey samples by ionic liquid-based cold-induced aggregation microextraction. *Talanta*, *155*, 216–221. https://doi.org/10.1016/j.talanta.2016.04.045

Wang, X., Anadón, A., Wu, Q., Qiao, F., Ares, I., Martínez-Larrañaga, M. R., Yuan, Z., & Martínez, M. A. (2018). Mechanism of neonicotinoid toxicity: Impact on oxidative stress and metabolism. *Annual Review of Pharmacology and Toxicology*, *58*, 471–507. https://doi.org/10.1146/annurev-pharmtox-010617-052429

Whitehorn, P. R., O'Connor, S., Wackers, F. L., & Goulson, D. (2012). Neonicotinoid pesticide reduces bumble bee colony growth and queen production. *Science*, *336*(6079), 351–352. https://doi.org/10.1126/science.1215025

Yamamuro, M., Komuro, T., Kamiya, H., Kato, T., Hasegawa, H., & Kameda, Y. (2019). Neonicotinoids disrupt aquatic food webs and decrease fishery yields. *Science*, *366*(6465), 620–623. https://doi.org/10.1126/science.aax3442