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Risk assessment of toxic effects of pesticide exposure in
wild terrestrial mammals

陸生野生哺乳類における農薬暴露による
毒性影響のリスクアセスメント

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ABBREVIATIONS

AAALAC: Association for Assessment and Accreditation of Laboratory

Animal Care International

ACP: Acetamiprid

ADI: Acceptable Daily Intake

BHC: Benzene Hexachloride

BLAST: Basic Local Alignment Search Tool

cDNA: Complementary DNA

CE: Collision energy

CLO: Clothianidin

CYP: Cytochrome P450

dc-ACP: Descyan-acetamiprid

DDW: Double-distilled water

DIN: Dinotefuran

d-SPE: Dispersive Solid Phase Extraction

dm-ACP: Desmethyl-acetamiprid

dpi: Days post infection

DZN: Diazinon

EMJH: Ellinghausen–McCullough–Johnson–Harris

GC/MS/MS: Gas Chromatography-Mass Spectrometry

HD: High dose

IFN: Interferon

IL: Interleukin

IMI: Imidacloprid

IP: Interferon-inducible protein

IU: International unit

KPB: Potassium phosphate buffer

K_m: Michaelis–Menten constant

LC/MS/MS: Liquid chromatography-mass spectrometry

LD: Low dose

MD: Medium dose

MDL: Method detection limit

MEGAX: Molecular Evolutionary Genetics Analysis X

MIP: Macrophage inflammatory protein

MRM: Multiple-Reaction Monitoring

N-ace-ACP: N-acetyl-acetamiprid

N-ace-DCP: N-acetyl-desmethyl-descyan-acetamiprid

N-ace-dm-ACP: N-acetyl-desmethyl-acetamiprid

nAChR: Nicotinic acetylcholine receptor

NCBI: National Center for Biotechnology Information

NIT: Nitenpyram

NIG: National Institute of Genetics

NNI: Neonicotinoid insecticide

PCR: Polymerase chain reaction

ppm: Parts per million

PQL: Practical Quantitative Limit

QC: Quality control

QuEChERS: Quick, easy, cheap, effective, rugged, and safe

RNA-seq: RNA sequencing

RNA: Ribonucleic acid

SD: Sprague Dawley

S/N: Signal to Noise ratio

SPE: Solid phase extraction

TCL: Thiacloprid

TGF: Transforming growth factor

THXM: Thiamethoxam

TNF: Tumor necrosis factor

TPM: Transcripts per million

Vmax: Maximum velocity

PREFACE

Necessity of wildlife conservation

“One World - One Health” is an important concept that the health of human, animal, and environmental are all strongly connected and we have to maintain all. Wildlife conservation is strongly needed as one of the approaches to “One Health.” There are many endangered species and keystone species that are highly important for maintaining the biodiversity and ecosystems. If the number of these important species decreases and the ecosystem changes, the ecosystem services that ecosystems provide to us may be reduced. The population of wildlife have been decreasing mainly by human activities such as environmental pollution, climate change, infectious diseases increased by artificial factors, habitat loss caused by development, impact of alien species, and poaching (Harfoot et al., 2021). The conservation of wildlife should be conducted under our responsibility and the benefits will help the survival of humans. Mammals are often species with large body sizes and large ecological linkages, or species that are apex predators and have a significant impact on ecosystems. Conservation of wild mammals is likely to lead to conservation of the environment as a whole.

Serious damage to wild mammals caused by pesticides

According to the IUCN Red Lists, the decline in wild mammal populations has been caused by multiple factors, mainly human activities. Chemical pollution is one of the most serious threats to wild mammals. Globally, agricultural fields have witnessed the widespread use of various pesticides, leading to their release into the environment

(Sharma et al., 2019; Tuji et al., 2021). Japan, for instance, has seen an annual influx of more than 230,000 tons of pesticides since 2018 (Chemical Database of the National Institute for Environmental Studies, Japan; https://www.nies.go.jp/kisplus/src_dvr/dvr). Pesticides are among the most influential xenobiotics with harmful effects on animals. Several negative effects including mass death of persistent and highly toxic pesticides, such as organochlorines and organophosphates, on wild mammals, were reported from a few decades ago (Grue et al., 1997; Kwon et al., 2004; Blus, 2011; Romanić et al., 2015). Although the usage of these compounds have decreased (Van Den Berg et al., 2017), novel pesticides such as neonicotinoid insecticides (NNIs) have been recently used in greater quantities (Gibbons et al., 2015; Pisa et al., 2021). Concerns have also been raised regarding the use of herbicides and fungicides, which can adversely affect biodiversity and wildlife populations. The alteration of vegetation structure by these chemicals can render habitats unsuitable for certain species, leading to significant declines in wildlife populations due to poisoning (Miller & Miller, 2003; Isenring, 2010).

In addition to pesticide exposure, infectious diseases are another major threat to wild mammals. Anthropogenic factors such as climate and environmental change have been reported to affect the infectious situation in wild mammals (Semenza & Paz, 2021; Baker et al., 2022). Wild mammals are exposed to a variety of pathogens, and the possibility that pesticide exposure may have some effect on them can be concerned.

Risk assessment of pesticide exposure in wild terrestrial mammals

It is important to properly assess the toxicity of pesticides in order to take action, but there are two major problems with current risk assessment of pesticides. First, there is a lack of pesticide toxicity testing using terrestrial wild mammals. The main targets of pesticide toxicity research are laboratory animals, livestock and humans, and there are not enough examples of research on wild mammals. In addition, the species subject to statutory toxicity testing are mainly aquatic animals such as fish and *Daphnia*, and few toxicity studies have been conducted on terrestrial wildlife. Secondly, the level of exposure of terrestrial wildlife to pesticides in the field is unknown. The degree of toxicity of a pesticide depends on the exposure dose. In aquatic areas, all animal species are exposed to the same environmental water and it is possible to calculate the estimated exposure based on parameters such as water volume and flow rate (Wang et al., 2024), but in terrestrial areas the mode of exposure and exposure concentration differ for each animal species, making exposure estimation even more complex and difficult than in aquatic areas. For terrestrial mammals, it is necessary to understand pesticide exposure not only in environmental samples but also at the individual species level, which is largely unknown.

In this study, we were able to elucidate insecticide exposure in terrestrial wildlife for the first time in Japan by establishing a simultaneous analytical method and sampling system for pesticides. In addition, *in vivo* tests using musk shrew as an animal model and evaluation of the effects of pesticide exposure on infectious disease were carried out. This research could be useful for appropriate risk management of pesticides, taking into account the conservation of wildlife and ecosystems.

Structure of this paper

This paper consists of four chapters.

Chapter 1: Estimation of the effects of neonicotinoid insecticides on wild raccoon, *Procyon lotor*, in Hokkaido, Japan: urinary concentrations and ability for hepatic metabolism of neonicotinoids

Chapter 2: Pesticide contamination levels in the stomach contents of wild raccoons (*Procyon lotor*) and masked palm civets (*Paguma larvata*) in Japan

The interaction between wildlife and environmental pollutants, in particular pesticides, has received increasing attention in recent years (Grue et al., 1997; Kwon et al., 2004; Blus, 2011; Romanić et al., 2015). In two parallel studies, I examined the effects of pesticide exposure on wildlife, focusing on the widespread use of NNIs and a wider range of pesticides in Japan. Chapter 1 focuses on NNIs, highlighting their emergence as ubiquitous environmental contaminants and their potential impact on wild mammals. I examined the exposure of raccoons in Hokkaido, Japan, to NNIs, shedding light on their urinary concentrations and hepatic metabolic capabilities. The study highlights the need to assess the susceptibility and toxicological effects of NNIs in wild mammalian species, providing insights that are critical for wildlife conservation efforts. Chapter 2 extends this investigation to a wider range of pesticides, reflecting on the widespread agricultural use of these chemicals and their impact on Japanese wildlife habitats. Focusing on raccoons and masked palm civets, I investigated pesticide concentrations in their stomach contents. This approach provides a comprehensive assessment of pesticide exposure levels and associated risks, highlighting the need to

understand the impact of pesticides on diverse wildlife ecosystems. Together, these studies contribute to our understanding of pesticide-induced toxicity in wild mammals and highlight the need for informed conservation strategies to mitigate the adverse effects of pesticide contamination on global biodiversity.

This content was announced in the following journal.

- Shinya, So, et al. “Estimation of the effects of neonicotinoid insecticides on wild raccoon, *Procyon lotor*, in Hokkaido, Japan: urinary concentrations and ability for hepatic metabolism of neonicotinoids.” *Environmental Toxicology and Chemistry*. 41(8). 2022: 1865-1874.

- Shinya, So, et al. “Pesticide contamination levels in the stomach contents of wild raccoons (*Procyon lotor*) and masked palm civets (*Paguma larvata*) in Japan.” *Environmental Toxicology and Chemistry*. 43(5) 2024: 943-951.

Chapter 3: Characteristics of cytochrome P450-dependent metabolism against acetamiprid in the musk shrew (*Suncus murinus*)

The complex relationship between environmental contaminants and wildlife health has received increasing attention in recent years (Grue et al., 1997; Kwon et al., 2004; Blus, 2011; Romanić et al., 2015). Shrews, as important members of the *Soricidae* family, play essential roles in soil ecosystems, yet their populations are in alarming decline worldwide (Kennerley et al., 2021; Schmidly et al., 2021). This decline, coupled with their potential exposure to pesticides (Mathias et al., 2007), underlines the urgency of understanding the toxicological implications for these species.

However, existing risk assessments predominantly focus on conventional laboratory animals, neglecting the unique physiological characteristics of shrews. This manuscript explores the metabolic intricacies of musk shrews and proposes them as a valuable model for assessing pesticide toxicity within the *Soricidae* family. Through pharmacokinetic, metabolic and genetic analyses, this study sheds light on the CYP-dependent metabolism of acetamiprid and provides insights into the toxicological landscape of neonicotinoid insecticides in shrews. This ground-breaking research provides the basis for tailored risk assessments that are essential to protect the health of these ecologically important species.

This content was announced in the following journal.

- Shinya, So, et al. "Characteristics of cytochrome P450-dependent metabolism against acetamiprid in the musk shrew (*Suncus murinus*).” *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*. 265. 2023: 109537.

Chapter 4: Effects of Diazinon Exposure on Urinary Shedding of *Leptospira interrogans* Serogroup Hebdomadis in Mice

Few studies have assessed the effects of pesticide exposure on pathogen reservoirs. Leptospirosis, a zoonotic disease, is a major global health concern due to its diverse manifestations in both humans and animals (Faine et al., 1999; Bharti et al., 2003). Understanding the dynamics of *Leptospira* shedding in reservoir animals such as rodents is crucial for assessing the risk of human infection (Costa et al., 2015; Barragan et al., 2017). I investigated the effect of DZN, a widely used insecticide (Aggarwal et al.,

2013; Abbasian et al., 2014), on urinary shedding of *Leptospira* in mice. Given the ubiquitous exposure of rodents to pesticides in the natural environment (Berny, 2007; Chi-Coyoc et al., 2016), this study addresses a previously unexplored area. By examining the interaction between DZN exposure, immune responses, histological changes in the kidneys and *Leptospira* shedding, this research sheds light on potential implications for human leptospirosis risk assessment. Through careful examination of urinary shedding, tissue analysis and cytokine gene expression, this study aims to contribute to a broader understanding of the effects of pesticide exposure on the transmission dynamics of infectious diseases.

This content was announced in the following journal.

- Shinya, So, et al. “Effects of diazinon exposure on urinary shedding of *Leptospira interrogans* serogroup Hebdomadis in mice.” *Toxics*. 11(4). 2023: 361.

CHAPTER 1

Estimation of the effects of neonicotinoid insecticides on wild raccoon, *Procyon lotor*, in Hokkaido, Japan: urinary concentrations and ability for hepatic metabolism of neonicotinoids

Abstract

Toxicological effects of 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. I considered that raccoons could be an animal model for evaluating NNIs-induced toxicities on wildlife as they inhabit near agricultural fields and eat crops treated with NNIs. The objective of this study was to estimate the effects of NNIs 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. I detected either 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 7 NNI compounds was 3.1 ng/mL. The urinary concentrations were not much different from those reported previously for humans. Furthermore, I performed an *in vitro* assessment of the ability of raccoons to metabolize NNIs using hepatic microsomes. The amounts of NNI metabolites were measured using LC/ESI/MS/MS and compared with those in rats. Raccoons showed much lower metabolic ability; the $V_{\max}/K_m$ values for raccoons were 1/10 to 1/3 of those for rats. For the first time, I show wild raccoons could be frequently exposed to NNIs in the environment, and the cytochrome P450-dependent metabolism of NNIs in the livers of raccoons might be low. This study would contribute to better understandings of the effects of NNIs on raccoons, leading to better conservation efforts for wild mammals.

Keywords

Neonicotinoid; Raccoon; Insecticide; Pesticide; Wildlife toxicology

Highlights

- Wild raccoons in Hokkaido, Japan, frequently show urinary concentrations of neonicotinoid insecticides (NNIs), suggesting ubiquitous exposure in the environment.
- Comparison urinary concentrations in raccoons with those in humans indicates similar exposure levels, except for acetamiprid, suggesting potential exposure risks to raccoons from agricultural practices.
- Hepatic metabolism assays show significantly lower metabolic abilities in raccoons compared to rats, suggesting increased susceptibility to NNI toxicity in raccoons.
- The results suggest that raccoons could serve as an indicator species for assessing the toxicological effects of NNIs on wild mammalian populations, highlighting the need for conservation efforts.

1. Introduction

Wild mammals are exposed to various environmental pollutants worldwide. Insecticides are among the most influential xenobiotics with harmful effects on animals. Several negative effects of persistent and highly toxic insecticides, such as organochlorines and organophosphates, on wild mammals, were reported from a few decades ago (Grue et al., 1997; Kwon et al., 2004; Blus, 2011; Romanić et al., 2015). However, the usage of these compounds have decreased (Van Den Berg et al., 2017). Recently, neonicotinoid insecticides (NNIs) have been getting more attention in the field of wildlife toxicology (Gibbons et al., 2015; Pisa et al., 2021). NNIs were developed and commercialized in the early 1990s and rapidly became some of the most frequently and widely used insecticides worldwide; they are used for several purposes in agriculture, veterinary medicine, and the residential environment (Simon-Delso et al., 2015). NNIs are nicotinic acetylcholine receptor (nAChR) 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 (ACP), imidacloprid (IMI), clothianidin (CLO), dinotefuran (DIN), thiacloprid (TCL), thiamethoxam (THXM), and nitenpyram (NIT), 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 (Chemical Database of National Institute for Environmental Studies, Japan, https://www.nies.go.jp/kisplus/src_dvr/dvr).

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 mortality in bee species compared with that in target insects, and exposure to NNIs is possibly one of the important factors for colony collapse disorder (Whitehorn et al., 2012; Lundin et al., 2015). 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 upon 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 alluded (Roberts et al., 2019).

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 NNIs detection from wild mammalian species (Byholm et al., 2018; MacDonald et al., 2018; Kaczyński et al., 2021). In these previous studies, NNIs have been detected in some organs such as the liver and muscle of some wildlife species such as 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 concentration 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. Additionally, susceptibility toward NNIs in wild mammals has not been investigated

(Pisa et al., 2021). 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 NNIs-induced toxicities, animal species with a high toxicological risk, such as highly sensitive species, should be used to avoid underestimating the toxic effects.

In this study, I 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 eat crops (Osaki et al., 2019). Although there are no previous reports about the NNIs exposure situation in raccoons, they could be exposed to NNIs after eating crops sprayed with NNIs. Additionally, 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 NNIs metabolism than other species. Owing to the possibilities of high exposure risk and low metabolic abilities, I 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 sacrificed in control programs to conserve the ecosystem, and I could obtain several samples of these sacrificed individuals.

In this study, I measured the urinary concentrations of NNIs to determine the exposure of wild raccoons in Hokkaido, Japan, and investigated their hepatic metabolic abilities to evaluate susceptibility toward these compounds.

2. Materials and Methods

2.1. Animals and treatments

Using box trap systems (Havahart Large Collapsible Pro Cage Model 1089, Woodstream Corp., Lititz, PA, USA), 54 raccoons were captured from a forest surrounded by cropland in Hokkaido, Japan, in 2021. Raccoons were collected from April to October when crops are generally cultivated outside, and NNIs are thought to be applied to crops; thus, sampling period was considered to be optimal for the assessment of NNIs exposure on raccoons. The captured raccoons were anesthetized in the box traps with 1.2 mg/kg butorphanol tartrate (Vetorphale, Meiji Seika, Tokyo, Japan), 40 µg/kg medetomidine hydrochloride (Dolbene, Kyoritsu, Tokyo, Japan), and 0.2 mg/kg midazolam (Dormicum, Astellas, Tokyo, Japan) administered through an intramuscular injection. Thereafter, raccoons were euthanized by injecting potassium chloride into their hearts. Five carcasses were collected as part of the feral raccoon control program in Hokkaido, Japan. Raccoons' body weight and sex were recorded, and their age was estimated using skulls (Junge & Hoffmeister. 1980). The age, sex, and date of capture are presented in Supplementary Table 1-1. 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 removed livers were immediately frozen in liquid nitrogen and preserved at -80°C until

the assay. All the 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 number: 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 (AAALAC).

2.2. Extraction of NNIs from urine samples

NNI compounds, including ACP, N-desmethyl-acetamiprid (dm-ACP), IMI, CLO, DIN, TCL, THXM, and NIT, were extracted and purified using a combination of QuEChERS and cold-induced aggregation microextraction methods (Vichapong et al., 2016; Song et al., 2019). First, 2 mL of urine sample was mixed with 2 mL of 16% sodium acetate buffer (0.5 g/3 mL double-distilled water [DDW]) and 4 mL of 1% formic acid in acetonitrile (ACN). Subsequently, 50 μ L of 100 ng/mL NNI internal standards containing deuterated labeled standards for ACP (ACP-d3), dm-ACP-(dm-ACP-d3), IMI (IMI-d4), CLO (CLO-d3), TCL (TCL-d4), THXM (THXM-d4), NIT (NIT-d3), and DIN (DIN-d3), were mixed by vortexing for 5 min. The mixtures were centrifuged at $10,000 \times g$ for 5 min and frozen overnight at -20°C . The supernatants (400 μ L) were filtered using CENTRICUT W-MO (Kurabo Industries, Osaka, Japan) by centrifuging at $12,000 \times g$ for 5 min. The refined samples were subjected to a solid phase extraction (SPE) method using ISOLUTE[®] HYDRO DME⁺ 400 mg/3 mL cartridge (Biotage, Uppsala, Sweden) (Suwannarin et al., 2020) and eluted with 600 μ L of ACN. After dry solidifying using a centrifugal evaporator (CVE-200D with UT-

2000; EYELA), extracts were redissolved in 200 μ L of 20% (v/v) methanol and transferred into vials for instrumental analysis.

Urine extract was analyzed using a liquid chromatography-mass spectrometer (6495 Triple Quadrupole LC/MS; Agilent Technologies, Santa Clara, CA, USA) equipped with a Kinetex Biphenyl column (3.0×100 mm, ϕ 2.7 μ m, Phenomenex Inc, Torrance, CO, USA). Electrospray ionization (ESI) was applied. The solvent, gradient, oven temperature, and flow rate used for the liquid chromatography are shown in Supplementary Table 1-2. Multiple-reaction monitoring (MRM) was used for mass spectrometry, as shown in Table 1-1. The analysis precision for all eight NNIs was confirmed by multiple analyses, as shown in Table 1-1. The analysis was performed using the internal standards methods. The information regarding the internal standards is provided in Supplementary Table 1-3. 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. NNIs standard was added to canine urines that did not contain any NNIs, and extraction and purification were performed in each calibration point using the described method. I 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 this study. The method detection limit (MDL) and accuracy (percent recovery) were determined by replicate analyses ($n = 5$) of NNI-mixed canine urine that did not contain any NNIs and NNI-mixed DDW at three different concentrations (1, 5, and 10 ng/mL). The practical quantitative limit (PQL) was settled as 5-times the MDL. The recovery rate and PQL are shown in Table 1-1. Urine samples from healthy dogs which no NNIs peak was detected were used for quality control (QC). A standard mixture of NNI compounds

was added to yield a urinary concentration of 5 µg/L. QC samples were injected with each batch of 20 samples. The between-day accuracy was within 80-120%.

2.3. 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 ACN. The sample was finally transferred into an HPLC vial for analysis using the LC/ESI/MS/MS (Shimadzu 20 A series with LCMS8040; Shimadzu) equipped with a Shodex Asahipak NH2P-40 2D (2 × 150 mm ID) column (Showa Denko America, Inc. NY, USA). The LC/ESI/MS analysis was carried out in an isocratic mode. Details for LC/ESI/MS/MS analysis, including solvent, gradient, oven temperature, flow rate, and selected *m/z* ions, are shown in Supplementary Table 1-4. 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.

2.4. Preparation of hepatic microsomes for in vitro NNI metabolism assay

Liver samples of adult raccoons (n=3) and 7-weeks old Sprague Dawley (SD) 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 above, and no NNIs

were detected from these raccoons. SD rats were purchased from Japan SLC, Inc. (Shizuoka, Japan). Hepatic microsomal fractions were prepared from raccoons and rats by 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 $9,000 \times g$ at 4°C for 20 min. The supernatants were filtered using gauze and centrifuged twice at $105,000 \times g$ at 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, IL, USA). The CYP concentration was determined using a reported method (Omura & Sato, 1964). The difference in the CO absorbance between 400 nm and 500 nm was examined by spectrophotometry (UV-2600; Shimadzu, Kyoto, Japan). The CYP concentration was calculated using the absorbance values at 450 nm and 490 nm.

2.5. *In vitro* CYP-dependent metabolism of NNIs

Metabolism assay of NNIs using hepatic microsomes was performed using a slight modification of the method described previously (Khidkhan et al., 2021). Different from the previously described method, the protein concentrations of liver microsome were changed from 5 mg/mL to 3 mg/mL for both raccoons and rats. The incubation mixture consisted of KPB (0.1 M, pH 7.4), MgCl₂ (3 mM in final concentration), and glucose-6-phosphate (5 mM), and hepatic microsomes (3 mg/mL of protein concentrations). As a substrate, IMI (Kanto Chemical Co., Inc., Tokyo, Japan), CLO (Wako Pure Chemical Co., Osaka, Japan), ACP (Cosmo Bio Co., Ltd., Tokyo, Japan), or THXM (Dr. Ehrenstorfer, Augsburg, Germany) in 3% methanol were added

at final concentrations of 10, 25, 50, 100, 200, and 400 μ M. 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 incubating for 30 min, 200 μ M of 1% formic acid in ACN was mixed to terminate the enzymatic reaction. Samples were then centrifuged at $15,000 \times g$ for 10 min, and the supernatant was filtered using a GL Chromato Disk sample filter (pore size, 0.2 μ M; GL Sciences, Tokyo, Japan). I performed all assays in duplicate for each concentration. Negative control mixtures without substrate were used for each sample.

An LC/ESI/MS/MS (6495 Triple Quadrupole LC/MS; Agilent Technologies, Japan) equipped with a Kinetex Biphenyl column (3.0×100 mm, ϕ 2.7 μ m, Phenomenex Inc, Germany) was used to measure the target metabolites of IMI, CLO, ACP, and THXM. In the previous study, following metabolites were produced by CYP-dependent *in vitro* metabolic assay in several mammalian species: 4OH-IMI, 5OH-IMI, desmethyl-CLO, and desmethyl-ACP (Khidkhan et al., 2021). Therefore, these metabolites were measured in this study: 4OH-IMI, 5OH-IMI, desmethyl-CLO (synthesized at Toho University, Chiba, Japan), and desmethyl-ACP (purchased from Sigma-Aldrich Co. LLC, Darmstadt, Germany) (Table 1-3). Details of LC/EIS/MS/MS analysis, including solvent, gradient, oven temperature, flow rate, collision energy (CE), and selected ions are shown in Supplementary Table 1-5.

2.6. Calculation of estimated daily intake amounts of NNIs from urinary concentrations

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

Estimated daily intake amount per body weight ($\mu\text{g}/\text{kg}/\text{day}$) = urinary NNIs concentration ($\mu\text{g}/\text{g}$ creatinine) $\times$ maximum daily creatinine excretion amounts per body weight in dog ($0.04 \text{ g}/\text{kg}/\text{day}$) $\times 1/r$ (urinary excretion correction coefficient)

The following values were applied for the urinary excretion correction coefficients: $r=0.586$ for ACP, $r=0.133$ for IMI, $r=0.596$ for CLO, $r=0.899$ for DIN, $r=0.05$ for TCL, $r=0.6$ for THXM (Ikenaka et al., 2019). These r values were obtained from previous studies on humans.

2.7. Statistical analysis

Statistical analyses were conducted using the JMP® Pro 16 software (SAS Institute). For the calculations of $\sum\text{NNIs}$, 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 version 8.0 for Windows (GraphPad Prism Software, CA, USA). The Student's t -test was performed to compare the $V_{\max}/K_m$ 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 the 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.

3. Results and Discussion

3.1. 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 1-2 and 1-3, respectively. Six parent NNI compounds (ACP, IMI, CLO, DIN, TCL, and THXM) and one NNI metabolite (dm-ACP) were detected in the following frequencies: CLO (66.1%) > dm-ACP (52.5%) > ACP (35.6%) > IMI (23.7%) > DIN (20.3%) > THXM (18.6%) > TCL (3.4%). NIT was not detected in all the raccoons. Either 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. This is the first study that detected NNI compounds in wild raccoons. Previously, NNI residues were found in wild terrestrial vertebrates, including wild boars, deer, and birds (MacDonald et al., 2018; Byholm et al., 2018; Kaczyński et al., 2021). Although NNIs were extracted from the blood and organs of wildlife species in these previous studies, I 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, Japan. In Japan, NNIs are often applied to agricultural crops, including corns and fruits, which are often eaten by raccoons (Osaki et al., 2019; Taira, 2014). It is possible that residues of NNIs applied for agriculture in the capture areas in this study might have transferred through crops into wild raccoons. The remains of crops were found in the stomachs of more than one-fourth of the raccoons in this study.

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 1-4). Creatinine-adjusted concentrations were used for comparison to correct for the hydration conditions of raccoons among spot samples as 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 applied to compensate for urinary levels of various compounds, including hormones and herbicides (Syme et al., 2007; Karthikraj & Kannan, 2019). Although there is no data on raccoons, the daily excreted amounts of creatinine in the urine are almost the same between dogs and humans (dogs: approximately 20–40 mg/kg/day; humans: approximately 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 in Carnivora; thus, dogs are much closer to raccoons than humans. Therefore, I 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 1-4). However, the results of ACP and NIT were different. Raccoons showed a significantly higher ACP concentration than humans (Mean value; raccoons: 0.28 µg/g creatinine, humans: 0.043 µg/g creatinine). In Japan, ACP is often applied to crops, such as vegetables and fruits (Fujita et al., 2012). Additionally, NIT was not detected in the raccoons in this study even though it was detected in humans at a rate of 30% (Ikenaka et al., 2019). The shipment amount of NIT in Hokkaido was the lowest of the seven NNI parent compounds in 2020 with only 5 tons per year, which is about 1.2% of the total shipment amount for the seven NNIs (Chemical Database of National Institute for Environmental

Studies, Japan. https://www.nies.go.jp/kisplus/src_dvr/dvr). Therefore, NIT might not have been applied to crops at the sampling site in this study.

Most of the exposed ACP is metabolized to dm-ACP and excreted through urine in mice (Ford & Casida, 2006). dm-ACP was more frequently and abundantly detected than ACP in raccoons, which suggests that this metabolite can be used as a marker of ACP exposure in wild raccoons as in humans (Marfo et al., 2015; Harada et al., 2016). In the future, it might be possible to detect NNIs with higher sensitivity by measuring various metabolites simultaneously. However, it has been confirmed that ACP in environmental soils is easily converted to dm-ACP by soil microorganisms (Rambhade et al., 2010). Therefore, direct exposure of dm-ACP to raccoons via environmental materials, such as soil and water in this study is also possible.

It should be noted that detected frequencies are not strictly comparable among each NNI compound because PQLs are different (ex. 0.03 µg/L for TCL and 0.29 µg/L for DIN). The detected frequency will tend to be higher as PQL gets lower. For strict comparison among NNI compounds, detected rates above PQL of DIN (0.29 µg/L and 0.33 µg/g creatinine), which had the highest PQL value, were calculated as shown in Tables 1-2 and 1-3. The detected frequencies were following: CLO (32.2%) > dm-ACP (20.3%) = DIN (20.3%) > ACP (8.5%) = IMI (8.5%) > THXM (6.8%) > TCL (1.7%). Apart from DIN, the order of the detected rates did not differ from when PQLs of each NNI was used. When PQL of DIN was used, the detected order of DIN was higher than when PQLs of each NNI were used. This indicates that it is important to apply common PQL value when I compare detected rates among multiple NNIs.

3.2. CYP-dependent metabolic ability of NNIs in the liver

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

Among all NNI metabolites analyzed in this study, raccoon microsomes showed much lower metabolic capacities than those of rats; $V_{\max}/K_m$ values in raccoons were approximately 1/10 to 1/3 of 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 the expression levels and functions of each CYP isoform. It is reported that NNIs such as CLO and THXM are metabolized by CYP 2A6, 2B6, 2C19, and 3A4 in humans (Shi et al., 2009). Although there is no report about CYP isoforms that metabolize ACP, 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.

3.3. 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 and 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 CLO, less than the no-observable-adverse-effect levels (NOAEL: about 50 mg/kg in 78-week carcinogenicity study) (Hirano et al., 2018). As shown in Table 1-6, daily intake amounts of NNIs were estimated from urinary concentration. The maximum estimated cumulative intake amount for the 6 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.

As 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 neuro-disruption, including increased frequency of anxiety-like behavior and decreased memory capacities in experimental animals (Hirano et al., 2015; Cimino et al., 2017).

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 this study were far lower than the exposure dose in previous animal experiments; generally, approximately 5-50 mg/kg of exposure is applied on mice and rats for the assessment of NNIs-induced neuro toxicities (Sheets et al., 2016). In wildlife species, non-lethal toxic effects that only have slight changes on individual levels can significantly impact on populations and ecosystem levels (Hay, 2009; Köhler & Triebskorn, 2013). 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.

5. Conclusions

In the present study, six parent NNI compounds and one NNI metabolite were detected in wild raccoons from Hokkaido, Japan. Either 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 showed not significantly different urinary concentrations as those in humans reported previously. The results of this study indicate that raccoons can be frequently exposed to NNIs in agricultural areas. Additionally, *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 1/10 to 1/3 of those for rats. This study 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.

Table 1-1: Selected neonicotinoids and their metabolites detected in raccoon urine samples.

Target neonicotinoids	PQL (µg/L)	Recovery rate (%)	RSD (%)	MRM (<i>m/z</i>)	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	+

PQL = practical detection limit, RSD = relative standard deviation, MRM = Multiple-reaction monitoring, CE = collision energy, ESI = electrospray ionization

Table 1-2: 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)	Percentiles (µg/L)					
				25th	50th	75th	95th	100th	PQL
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
Σ 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).

PQL = practical detection limit, GM = geometric mean

Σ 8NNIs = sum of creatinine-adjusted concentrations of neonicotinoid compounds

Table 1-3: 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)	Percentiles (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
Σ 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).

PQL = practical detection limit, GM = geometric mean

Σ 8NNIs = sum of creatinine-adjusted concentrations of neonicotinoid compounds

Table 1-4: Urinary neonicotinoid concentrations (microgram per gram creatinine) in raccoons compared to that in Japanese people.

Subject		ACP	dm-ACP	IMI	CLO	DIT	TCP	TMXM	NIT
Raccoons	DF (%)	35.59	52.54	23.73	66.1	20.34	3.39	18.64	0
Present study (n = 59)	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	DF (%)	11.11	86.67	17.78	48.89	48.89	33.33	33.33	26.67
(Ikenaka et al., 2019)	Mean	0.043	2.2	0.13	1.06	0.57	0.03	0.17	0.1
(n = 45)	Maximum	1.03	26.92	1.59	15.06	9.49	0.2	2.11	1.25

DF = detection frequency. ACP = acetamiprid, dm-ACP = dimethyl acetamiprid, IMI = imidacloprid, CLO = clothianidin, DIT = dinotefuran, TCP = thiacloprid, TMXM = thiamethoxam, NIT = nitenpyram

Table 1-5: Michaelis–Menten kinetics parameters ($V_{\max}$ (pmol/min/mg), K_m (μM), $V_{\max}/K_m$ ($\mu\text{L}/\text{min}/\text{mg}$), mean $\pm$ SD) for cytochrome P450 (CYP) metabolism of neonicotinoids.

Substrate	Metabolite		Rat	Raccoon	Remark
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 1-6: Estimated daily intake amounts of NNIs from urinary concentrations ($\mu\text{g/kg/day}$) in raccoons (n = 59).

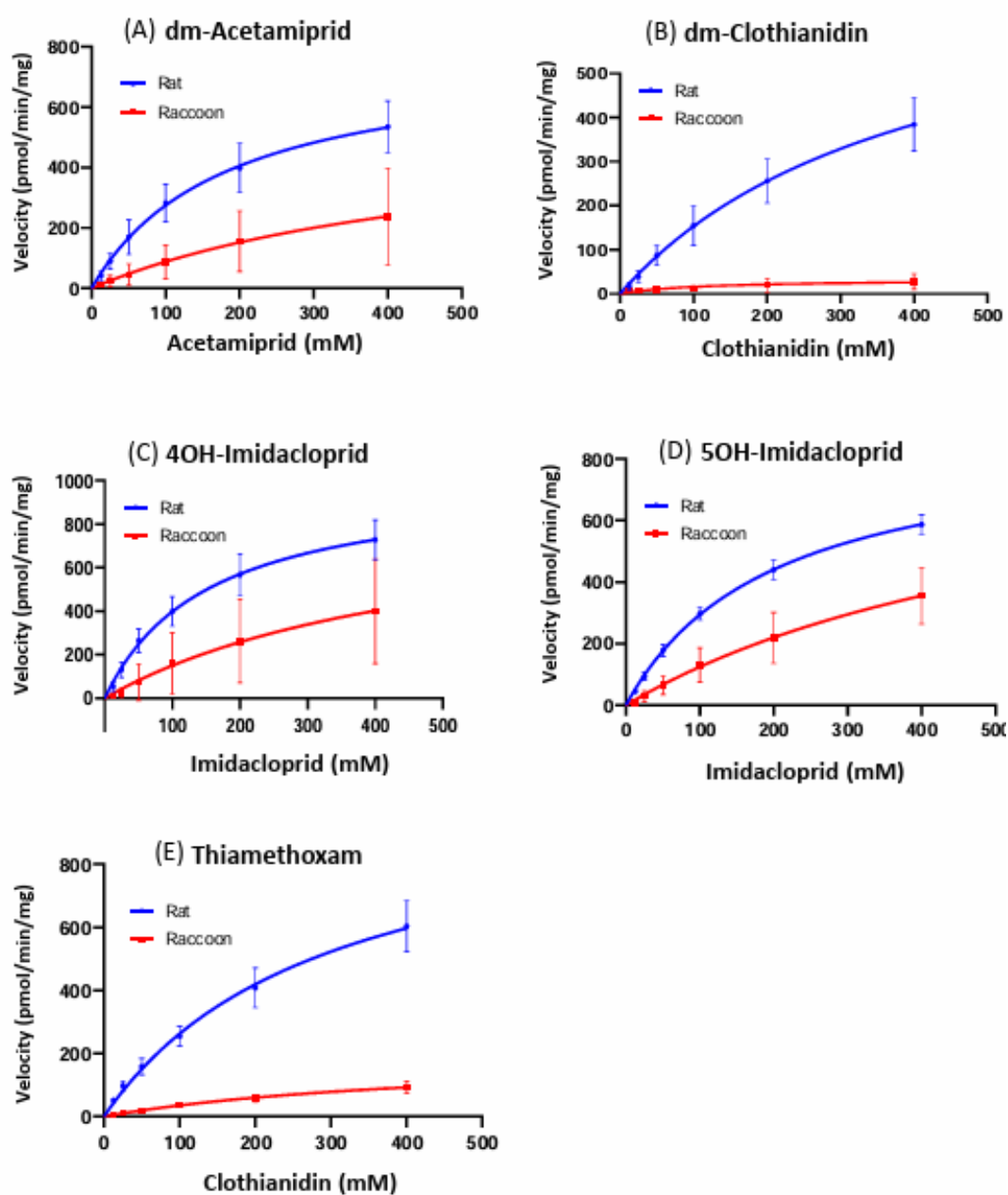
Neonicotinoid	GM $\pm$ SD ($\mu\text{g/kg/day}$)	Percentiles ($\mu\text{g/kg/day}$)				
		25th	50th	75th	95th	100th
Acetamiprid	0.019 $\pm$ 0.051	<PQL	<PQL	0.017	0.054	0.383
Imidacloprid	0.069 $\pm$ 0.225	<PQL	<PQL	<PQL	0.129	1.731
Clothianidin	0.054 $\pm$ 0.111	<PQL	0.014	0.029	0.268	0.631
Dinotefuran	0.033 $\pm$ 0.073	<PQL	<PQL	<PQL	0.061	0.457
Thiacloprid	0.075 $\pm$ 0.349	<PQL	<PQL	<PQL	<PQL	2.729
Thiamethoxam	0.019 $\pm$ 0.063	<PQL	<PQL	<PQL	0.033	0.464
Σ 6 NNIs	0.269 $\pm$ 0.713	0.065	0.113	0.213	0.630	5.513

PQL = practical detection limit, GM = geometric mean

Σ 6 NNIs = sum of estimated daily intake amounts of neonicotinoid compounds

Figure 1-1: Michaelis–Menten plots for cytochrome P450 (CYP) activity of neonicotinoid insecticides in SD rat (blue circle) and raccoon (red square) liver microsomes.

Each value is shown as mean $\pm$ SD.



Supplementary data

Table S1-1. Age, sex, and date of capture of raccoons used in this study.

Table S1-2. Detailed parameters of liquid chromatography for detecting urinary NNI concentrations.

Table S1-3. Internal standards of neonicotinoids used in this study.

Table S1-4. Detailed parameters of liquid chromatography for detecting urinary creatinine concentrations.

Table S1-5. Detailed parameters of liquid chromatography for *in vitro* metabolic assay.

Table S1-1. Age, sex, and date of capture of raccoons used in this study.

Date of capture	Sex	Body weight (kg)	Age
March 27	♂	2.86	Cub
March 28	♂	2.66	Cub
March 28	♂	5.66	Adult
March 31	♂	5.54	Cub
April 3	♂	4.02	Cub
April 9	♂	7.02	Adult
May 6	♂	4.86	Cub
May 7	♂	7.52	Adult
May 8	♂	5.94	Cub
May 9	♀	4.52	Adult
May 9	♂	8.00	Adult
May 10	♂	4.40	Cub
May 10	♂	4.54	Cub
May 11	♂	6.00	Adult
May 11	♀	4.62	Adult
May 13	♂	5.30	Cub
May 14	♂	3.64	Cub
May 15	♂	3.26	Cub
May 18	♂	3.96	Cub
May 21	♂	5.30	Cub
May 23	♂	5.96	Cub
May 23	♀	6.30	Adult
May 26	♂	6.70	Adult
May 27	♂	7.62	Adult
May 27	♂	6.28	Cub
May 31	♂	5.16	Cub
June 2	♂	4.04	Cub
June 2	♀	4.28	Adult
June 3	♀	5.60	Adult
June 9	♂	5.22	Adult
June 9	♂	5.98	Adult
June 9	♂	6.46	Cub
June 16	♂	6.46	Adult

June 16	♂	4.90	Cub
June 22	♂	3.20	Cub
June 22	♂	1.94	Cub
June 23	♀	3.90	Adult
June 23	♂	1.70	Cub
June 23	♀	6.86	Adult
June 23	♀	6.6	Adult
June 26	♂	1.88	Cub
June 26	♂	7.00	Adult
June 30	♂	9.4	Adult
July 1	♀	5.04	Adult
July 1	♂	4.70	Adult
July 3	♂	4.74	Adult
July 12	♂	0.84	Cub
July 12	♂	0.64	Cub
July 13	♀	7.44	Adult
September 1	♂	6.10	Adult
September 6	♂	6.46	Adult
September 6	♂	5.70	Adult
September 8	♂	4.24	Cub
September 9	♂	4.50	Cub
September 9	♂	4.26	Cub
October 5	♂	4.70	Cub
October 9	♂	3.88	Cub
October 25	♂	9.06	Adult
October 29	♂	5.92	Cub

Cub: younger than 1 year old.

Adult: older than 1 year old.

Table S1-2. Detailed parameters of liquid chromatography for detecting urinary NNI concentrations.

Solvent A	0.1% (v/v) formic acid and 10 mM ammonium acetate in DDW	
Solvent B	0.1% (v/v) formic acid and 10 mM ammonium acetate in methanol	
Column oven	60°C	
Flow rate	0.7 mL/min	
Gradient		
Time (min)	Solvent A (%)	Solvent B (%)
0-1	98	2
12	35	65
12-14	2	98

Table S1-3. Internal standards of neonicotinoids used in this study.

Target compounds	RSD (%)	MRM (<i>m/z</i>)	CE	Polarity for ESI
Acetamiprid d3	6	226.7>126.2	24	+
N-dm-Acetamiprid d3	8	212.2>73.1	60	+
Imidacloprid d4	4	260.1>179.1	24	+
Clothianidin d3	6	253.0>171.9	8	+
Dinotefuran d3	6	206.1>132.1	12	+
Thiacloprid d4	7	257.1>90.1	50	+
Thiamethoxam d4	5	296.1>185.2	24	+
Nitenpyram d3	9	274.1>228.2	8	+

PQL = practical detection limit, RSD = relative standard deviation,
MRM = Multiple-reaction monitoring, CE = collision energy, ESI = electrospray ionization

Table S1-4. Detailed parameters of liquid chromatography for detecting urinary creatinine concentrations.

Solvent A	50 mM ammonium formate in DDW
Solvent B	acetonitrile
Concentration of solvent B: 90 %	

Column oven	45°C
Flow rate	0.4 mL/min

Target	MRM (<i>m/z</i>)	Polarity for ESI
Creatinine	113.9 > 43	+
Creatinine-d3	116.9 > 47.1	+

MRM = Multiple-reaction monitoring, ESI = electrospray ionization

Table S1-5. Detailed parameters of liquid chromatography for *in vitro* metabolic assay.

For dm-CLO, dm-ACP, and CLO				For 4OH-IMI and 5OH-IMI		
Solvent A	0.1% formic acid + 10 mM ammonium acetate in DDW			Solvent A	DDW	
Solvent B	0.1% formic acid + 10 mM ammonium acetate in methanol			Solvent B	0.1% formic acid + 10 mM ammonium acetate in methanol	

Target	MRM (<i>m/z</i>)	CE	Polarity for ESI
4OH-IMI	272.0 > 191.2	24	+
5OH-IMI	272.0 > 191.2	16	+
dm-CLO	236.0 > 132.0	16	+
dm-ACP	209.1 > 125.8	20	+
CLO	250.0 > 132.0	12	+

Gradient		
Time (min)	Solvent A (%)	Solvent B (%)
0-1	98	2
8	20	80
8-9.5	2	98

Column oven	60°C
Flow rate	0.35 mL/min
Injection volume	5 µL

MRM = Multiple-reaction monitoring, CE = collision energy, ESI = electrospray ionization

CHAPTER 2

Pesticide contamination levels in the stomach contents of wild raccoons (*Procyon lotor*) and masked palm civets (*Paguma larvata*) in Japan

Abstract

Pesticides, vital for agriculture, pose a significant threat to wildlife in transformed Japanese landscapes. Despite global reports of pesticide poisoning in animals, limited studies have examined current wildlife exposure in croplands or metropolitan areas in the region. Using liquid chromatography-mass spectrometry (LC/MS/MS) and gas chromatography-mass spectrometry (GC/MS/MS), this study aimed to assess the contamination status of 368 commonly used pesticides. The stomach contents of raccoons living in croplands contained 13 pesticides, including six herbicides and 11 fungicides. Neonicotinoid insecticides, some fungicides, and previously banned insecticides (benzene hexachloride and dichlofenthion) were most frequently detected and found at the highest concentrations, suggesting direct soil-plant transfer and direct consumption by crop-eating species. In masked palm civets living in metropolitan areas, four insecticides and six fungicides were detected, indicating urban wildlife exposure from raided dustbins, urban gardens, and lumber from houses. Although the maximum measured concentrations of all pesticides were lower than the acceptable daily intake for humans, it remains unclear whether these concentrations may have toxic or adverse health effects on the evaluated species in these transformed landscapes. This study is the first to examine recent pesticide exposures in wild mammals in Japan. Additionally, the application of the method developed in this study will lay the foundation for the examination of pesticides in other wildlife species to assist conservation management efforts in the region.

Keywords

Insecticide; Herbicide; Fungicide; Pesticide; Pollutant; Wildlife

Highlights

- Raccoons in croplands were found to be exposed to various pesticides, particularly neonicotinoid insecticides, at levels comparable to those found in urine samples.
- Detection rates of certain pesticides differed significantly between raccoons with and without confirmed corn ingestion, highlighting corn as a substantial contributor to pesticide exposure.
- Masked palm civets in metropolitan areas showed high detection rates of industrial antifungal agents, likely due to frequent exposure to treated wood, and neonicotinoid insecticides from raided dustbins and urban gardens.
- The estimated maximum exposure levels of the pesticides detected in both species were below the acceptable daily intake for humans, but potential synergistic effects and acute toxicity risks warrant further investigation.

1. Introduction

Globally, agricultural fields have witnessed the widespread use of various pesticides, leading to their release into the environment (Sharma et al., 2019; Tudi et al., 2021). Japan, for instance, has seen an annual influx of more than 230,000 tons of pesticides since 2018 (Chemical Database of the National Institute for Environmental Studies, Japan; https://www.nies.go.jp/kisplus/src_dvr/dvr). The consequential adverse effects of pesticides on wildlife have been reported worldwide. For example, dichloro-diphenyl-trichloroethane has been implicated in the mass mortality of wild birds (Blus, 2011), while cases of poisoning stemming from various organophosphorus and carbamate insecticides have been documented in wild mammals (Botha et al., 2015; Ruiz-Suarez et al., 2015). Concerns have also been raised regarding the use of herbicides and fungicides, which can adversely affect biodiversity and wildlife populations. The alteration of vegetation structure by these chemicals can render habitats unsuitable for certain species, leading to significant declines in wildlife populations due to poisoning (Miller & Miller, 2003; Isenring, 2010). Despite these concerns, studies examining the exposure of wildlife to currently applied pesticides in Japan are lacking, with a particularly scarce body of research focused on wild mammals. To our knowledge, only one study has investigated recent pesticide exposure in Japanese wild mammals (chapter 1; Shinya et al., 2022). Given the imperative of wildlife conservation in Japan, there is a crucial need to understand the extent of pesticide exposure in Japanese wildlife habitats and ecosystems.

In this study, I focused on raccoons (*Procyon lotor*) from Hokkaido, Japan, and masked palm civets (*Paguma larvata*) from Tokyo, Japan, as wildlife models inhabiting croplands and metropolitan areas, respectively. Within the survey area of Hokkaido,

raccoons feed on crops (Osaki et al., 2019), while masked palm civets in Tokyo predominantly consume leftover food and garbage (Matsuo & Ochiai, 2009; Iwama et al., 2017). Consequently, each species is likely exposed to pesticides by directly ingesting food items comprising their diet.

Notably, neonicotinoid insecticides have been detected in raccoon urine in our chapter 1. Given that raccoons and masked palm civets are recognized as invasive alien species in Japan and control programs have been implemented (Iwama et al., 2017; Suzuki & Ikeda, 2019), I was able to obtain fresh samples for analysis. Considering the potential for pesticide exposure due to their feeding habits and the feasibility of sample collection, raccoons and masked palm civets were selected as model species inhabiting agricultural and urban areas, respectively.

In animals exposed to pesticides, these substances are absorbed in the intestine after passing through the stomach and undergoing metabolism in the liver and kidneys, ultimately being excreted through urine and feces (Barton et al., 2006). Due to the metabolization of pesticide compounds, urine and feces typically contain higher concentrations of metabolites than their parent compounds (Aprea et al., 2002; Barton et al., 2006). In contrast, analyzing stomach contents enables the detection of parent compounds, thereby facilitating the direct assessment of pesticide exposure. The objectives of this study were to i) quantify the pesticide concentrations in the stomach contents of raccoons and masked palm civets using liquid chromatography-tandem mass spectrometry (LC/MS/MS) and gas chromatography-tandem mass spectrometry (GC/MS/MS), and ii) evaluate the exposure levels of each pesticide and the associated risk of pesticide-induced toxicities in raccoons and masked palm rivets. The utilization of LC/MS/MS enables the detection of compounds spanning a wider range of polarities,

while GC/MS/MS is effective in analyzing highly volatile compounds, a capability not applicable to LC/MS/MS measurements (Raina, 2011; He & Age., 2019). Capitalizing on these complementary advantages and acknowledging the limitations of each method, I incorporated both LC/MS/MS and GC/MS/MS in this study.

2. Materials and Methods

2.1. Animals and treatments

Animals and treatments

Forty-one raccoons were captured from croplands in Hokkaido, Japan, between April 2021 and September 2022, utilizing box trap systems (Havahart Large Collapsible Pro Cage Model 1089, Woodstream Corp., Lititz, PA, USA), as described in chapter 1. Anesthesia was administered to the raccoons through a combination of butorphanol tartrate (Vetorphale, 1.2 mg/kg; Meiji Seika, Tokyo, Japan), medetomidine hydrochloride (Dolbene, 40 µg/kg; Kyoritsu, Tokyo, Japan), and midazolam (Dormicum, 0.2 mg/kg; Astellas, Tokyo, Japan) via intramuscular injection. Subsequently, euthanasia was performed by injecting potassium chloride into their hearts. The age of the raccoons was estimated based on skull characteristics, following the method outlined by Junge and Hoffmeister (1980).

Thirty carcasses of masked palm civets, captured in box traps between September 2021 and September 2022 in highly urbanized areas of Tokyo, Japan (Minato, Toshima, Edogawa, Shibuya, and Suginami wards), were provided by the Tokyo Pest Control Association. Details, including capture date, sex, body weight, and approximate estimated age of each raccoon and masked palm civet, are shown in Tables S2-1 and S2-2, respectively. The dissection of raccoons and masked palm civets was

conducted using disposable surgical knives, with more than 3 ml of stomach contents collected from each specimen using disposable chopsticks. Only solid materials were sampled, excluding liquids. To prevent cross-contamination, distinct surgical knives and chopsticks were allocated for each individual. The collected samples were then divided for food type identification and pesticide extraction. After thorough mixing using disposable chopsticks to ensure even distribution of any contained food item, samples were placed in two 50 mL conical centrifuge tubes. All samples were stored at -20°C until further analysis.

All procedures involving animal handling and experiments strictly adhered to the guidelines set forth by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Approval number: 18-0001). The animal experiments conducted at the Graduate School of Hokkaido University adhered to the guidelines outlined by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.2. Identification of food types in the stomach contents of raccoons and masked palm civets

The analysis of stomach contents from each animal followed the methods outlined by Osaki et al (2019). In summary, 0.5 g of defrosted sample was placed on a 0.5 mm mesh sieve and washed with tap water. The solid material retained on the sieve was then spread into a laboratory dish and categorized into six groups for qualitative analysis: wild berries and seeds, plants (including herbaceous plants and woody forbs), crops (such as corn, rice, potato, and apple), birds, reptiles, and insects. Items such as groomed hair, artificial materials (plastic, rubber, and cloth), stones, and gravel were

excluded from the analysis. These exclusions were made as the items were likely unintentionally consumed by the animals during their time in the box trap after capture, as observed in a previous study by Suld et al. (2014). In a preliminary test involving ten raccoons with no crops but only groomed hair, artificial materials, stones, and gravel, pesticide concentrations were measured using the same extraction and mass spectrometry method described below. The results revealed no detection of pesticides in these ten raccoons (data not shown).

2.3. Extraction of pesticides from the stomach contents of raccoons and masked palm civets

Pesticides were extracted and purified from the stomach contents using a combination of QuEChERS and dispersive solid phase extraction (d-SPE) methods (Lawal et al., 2018; Reis et al., 2020). Approximately 2 g of the stomach contents were shredded using scissors and mixed with 4 mL of 1% formic acid in acetonitrile and 4 mL of acetate buffer (0.5 g/3 mL double distilled water (DDW)). Subsequently, 200 μ L of a 200 ppb internal standard mixture containing six compounds for LC/MS/MS analysis (acetamiprid-d₃, clothianidin-d₃, thiacloprid-d₄, thiamethoxam-d₄, nitenpyram-d₃, and dinotefuran-d₃; Hayashi Pure Chemical Ind., Ltd., Osaka, Japan) and 11 compounds for GC/MS/MS analysis (dichlorvos-d₆, chlorpyrifos-d₁₀, diethofencarb-d₇, isoprothiolane-d₄, isoxathion-d₁₀, ethyl-nitrophenyl-phenyl phosphonothioate-d₅, ethofenprox-d₅, fenvalerate-d₇, acephate-d₆, carbaryl-d₇, and metolachlor-d₆; Hayashi Pure Chemical Ind., Ltd., Osaka, Japan) was added. The samples were thoroughly mixed by vortexing for 5 min. Thereafter, 3 g of magnesium sulfate and 0.75 g of sodium acetate (Q-sep QuEChERS Extraction Kit, RESTEK, PA,

USA) were added and mixed vigorously. Subsequently, the mixtures were centrifuged at $10,000 \times g$ for 10 min. After centrifugation, 1 mL of each supernatant transferred to Q-sep QuEChERS d-SPE 2 mL tubes containing 150 mg magnesium sulfate and 50 mg C18 (RESTEK) were vortexed and centrifuged at $10,000 \times g$ for another 10 min. Following this centrifugation, 600 μ L of the supernatant was filtered using a GL Chromato Disk (pore size, 0.2 μ m; GL Sciences, Tokyo, Japan) and transferred to two test tubes (300 μ L each). Subsequently, the samples underwent dry-solidification under a gentle nitrogen stream (MGS-HEAT2; Tokyo Rikakikai Co., Ltd.) and were redissolved in 300 μ L of 20% (v/v) methanol or acetone for LC/MS/MS or GC/MS/MS analysis, respectively. The samples were then centrifuged at $15000 \times g$ for 10 min, and the resultant supernatants were subjected to LC/MS/MS and GC/MS/MS analyses.

2.4. Stock solutions, calibration standards, and quality control

Standard mixtures of pesticides, namely PL2005 Pesticides LC/MS/MS Mix 1 to 12 and PL2005 Pesticides GC/MS/MS Mix 1 to 7, were purchased from Hayashi Pure Chemical Ind., Ltd. (Osaka, Japan). Stock solutions were meticulously prepared by dissolving the mixtures in suitable solvents (methanol for the LC/MS/MS mix and acetone for the GC/MS/MS mix) to achieve a concentration of 2 μ g/mL. These stock solutions were carefully stored at -20°C until further use. Intermediate working solutions for LC/MS/MS and GC/MS/MS were prepared by combining individual standards, resulting in a concentration of 100 ng/mL for each pesticide. These working solutions were also stored at -20°C . The pesticides contained in the prepared solutions are detailed in Tables S2-3 and S2-4.

For each compound, calibration curves were constructed using final concentrations of 0.5, 1, 2.5, 5, 10, 25, 50, and 100 ng/mL. Procedural blank samples were prepared with each batch of 20 samples. Stomach content samples from raccoons, where no pesticide peak was detected, were utilized for the analysis of parameters such as the method detection limit (MDL), accuracy (percent recovery), and quality control (QC). MDL and recovery rate were determined through replicate analyses ($n = 5$) of pesticide standard-mixed stomach contents at three concentrations (1, 5, and 25 ng/mL). The practical quantitative limit (PQL) was set at five times the MDL. The recovery rate and PQL for LC/MS/MS and GC/MS/MS analyses are detailed in Tables S2-3 and S2-4, respectively. For QC analysis, the standard mixture of target pesticides was blended with stomach contents to yield a concentration of 5 ng/mL. QC samples were injected with each batch of 20 samples. The between-day accuracy was maintained within the range of 80–120%.

2.5. LC/MS/MS analysis conditions

The stomach content extracts were analyzed using LC/MS/MS (6495 triple quad LC/MS; Agilent Technologies, Santa Clara, CA, USA) equipped with the Agilent ZORBAX Eclipse Plus C18 RRHD column (2.1×100 mm, $\phi 1.8$ μ m, Agilent Technologies). The samples were subjected to gradient elution; the mobile phase comprised 2 mM ammonium acetate as solvent A in DDW and 100% methanol as solvent B. The following gradient was applied: $t = 0$ min, 5% solvent B; $t = 27$ min, 100% solvent B; $t = 27$ –35 min, 100% solvent B; and $t = 35$ –37 min, 5% solvent B. The flow rate was set as follows: $t = 0$ –33 min, 0.2 mL/min; $t = 33$ –37 min, 0.4 mL/min. The column oven temperature was maintained at 40°C, and the injection volume was 5 μ L.

For mass spectrometry, multiple-reaction monitoring (MRM) was employed (Table S2-3). The analysis was performed using the internal standard method. In cases where target pesticides lacked a corresponding internal standard, the concentration was corrected using the internal standard with the closest recovery rate.

2.6. GC/MS/MS analysis conditions

The stomach content extracts were analyzed using GC/MS/MS (GCMS-TQ8050 NX, triple quad GC/MS; Shimadzu Corporation, Kyoto, Japan) equipped with the SH-I-5Sil MS capillary column (length: 30 m; inner diameter: 0.25 μ m; film thickness: 0.25 μ m; Shimadzu Corporation). Helium was served as the carrier gas for GC analysis, with gas pressure, column flow rate, linear velocity, and split ratio set at 100 kPa, 1.7 mL/min, 47.2 cm/s, and -1.0, respectively. The column temperature was initially set at 50°C for 1 min and then increased to 125°C at a rate of 25°C/min. Subsequently, the temperature was raised to 300°C at a rate of 10°C/min and held at 300°C for 15 min. For mass spectrometry, the injection volume was 3 μ L, the ion source temperature was 230°C, the interface temperature was 250°C, and the detector voltage was 0.6 kV. MRM was employed, and details are provided in Table S2-4. The analysis followed the internal standard method. In cases where target pesticides lacked a corresponding internal standard, the concentration was adjusted using the internal standard with the closest recovery rate.

2.7. Comparison of detectable rates of neonicotinoid insecticides between stomach contents and urine samples of raccoons

This study aimed to evaluate the presence of neonicotinoid insecticides in the stomach contents of raccoons in the Hokkaido region of Japan. This assessment was conducted to compare the neonicotinoid insecticide concentrations previously quantified in urine samples collected from raccoons in the same region as described in chapter 1.

2.8. Estimation of maximum exposure levels of pesticides

Based on the concentrations of specific pesticides detected in the stomach contents of raccoons and masked palm civets, a species-specific assessment of current pesticide exposure was conducted. This estimation considered the maximum stomach content capacity of a medium-sized domestic dog (90 mL/kg; Hodson & Tobias, 2014), a size comparable to both species. The specific gravity of most crops (~1.0 g/mL; Dowdy & Larson, 1975; Annunciado et al., 2005) was employed to set the maximum stomach capacity for both species on a crop-only diet at 90 g/kg (90 mL/kg × 1.0 g/mL). The calculation of the estimated maximum exposure of raccoons and masked palm civets to each pesticide, indicative of exposure levels when animals consume only crops contaminated with pesticides, was performed using the formula:

Estimated maximum exposure level (µg/kg) = detected level of pesticide in stomach contents (ng/g) × estimated capacity of the stomach (90 g/kg) × 1/1000

To evaluate the species-specific risk of pesticide-induced toxicity, these estimated maximum exposure levels were compared with data on the acceptable daily intake (ADI) for humans obtained from <https://pubchem.ncbi.nlm.nih.gov/>.

2.9. Statistical analyses

To calculate the sum of detected pesticide concentrations (Σ pesticides), values below the PQL were set to zero. For statistical significance analysis, concentrations of pesticides below the PQL were imputed as the PQL value divided by the square root of 2 (Hornung & Reed, 1990). To identify food types, the frequency of occurrence for each food category was calculated as a percentage using the following formula:

Frequency of occurrence (%) = number of animals with the food category in their stomach contents / number of animals used in this study (n = 41 for raccoons; n = 30 for masked palm civets)

Statistical analyses were performed using the JMP® Pro 16 software (SAS Institute). The Shapiro–Wilk test and Levene’s test were employed to assess the normal distribution and homogeneity of variance, respectively. Using the Wilcoxon test, Σ pesticides and concentrations of pesticides with a detection rate of more than 30% were subjected to the following comparisons: 1) male and female, 2) adult and cub, and 3) animals in which crops were detected in their stomach contents and those in which no crops were detected. A *p*-value of < 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Food types in the stomach contents

A comparison of food types and their frequency of occurrence in the stomach contents of raccoons and masked palm civets (Table 2-1) revealed that corn was the sole crop identified in raccoons, constituting 51.2% of the diet. In contrast, apple, rice, and potato crops occurred individually or in combination at frequencies of 23.3%, 13.3%, and 10%, respectively, in at least 40% of all masked palm civets examined.

3.2. Pesticide concentrations in the stomach contents of raccoons and masked palm civets

Pesticide concentrations detected in the stomach contents of raccoons revealed the presence of several pesticides with a detection rate exceeding 30% (Table 2-2). Carbendazim exhibited the highest detection rate (70.73%), followed by tricyclazole (56.10%), imidacloprid (41.46%), acetamiprid (39.02%), and clothianidin (36.59%). Raccoons with confirmed corn in the stomach contents showed significantly higher Σ pesticides ($p = 0.034$), as well as concentrations of acetamiprid ($p = 0.010$), clothianidin ($p = 0.038$), and tricyclazole ($p = 0.041$), compared to raccoons without confirmed corn in the stomach contents. However, no significant difference was observed in pesticide concentrations based on the sex and age of the raccoons.

When comparing pesticide detection rates in stomach contents from this study with urine samples from chapter 1, the following trends were consistent in both matrices: higher detection rates of following neonicotinoid compounds than others, such as imidacloprid (41.46% in stomach contents vs. 23.37% in urine), acetamiprid (39.02% vs. 35.59%), and clothianidin (36.59% vs. 66.10%), followed by thiamethoxam (12.20% vs. 18.64%); however, both thiacloprid (0% vs. 3.39%) and nitenpyram (0% vs. 0%) were not detected in the stomach contents (Table 2-3). Regarding dinotefuran (2.44% vs. 20.34%), stomach contents exhibited a notably low detection rate, however in urine, its detection rate was comparable to that of imidacloprid (23.37%).

The concentrations of pesticides in the stomach contents of masked palm civets are detailed in Table 2-4. Among the pesticides detected in masked palm civets, those with >30% detection rates included carbendazim (76.67%), thiabendazole (70.00%),

acetamiprid (43.33%), and thiacloprid (33.33%). No significant differences were observed in Σ pesticides and the concentrations of highly detected pesticides in relation to sex, age, and the presence of crops in the stomach contents.

A comparison of the results between raccoons and masked palm civets revealed substantial differences in the detection rates of certain pesticides. Clothianidin exhibited a 3.66-fold difference (36.59% in raccoons vs. 10.00% in masked palm civets), imidacloprid showed a 6.22-fold difference (41.46% in raccoons vs. 6.67% in masked palm civets), thiacloprid was only detected in masked palm civets (0% raccoons vs. 33.33% in masked palm civets), thiamethoxam was only detected in raccoons (12.20% in raccoons vs. 0% in masked palm civets), thiabendazole exhibited a 9.56-fold difference (7.32% in raccoons vs. 70.00% in masked palm civets), and tricyclazole showed a 5.61-fold difference (56.10% in raccoons vs. 10.00% in masked palm civets).

3.3. Estimation of maximum exposure levels of pesticides

Utilizing the pesticide concentrations in the stomach contents of both species, the maximum exposure levels were estimated and compared with the ADI for humans. Across all pesticides evaluated, the estimated maximum exposure levels remained below the established ADI for humans (Tables 2-2 and 2-4).

4. Discussion

4.1. Pesticides detected in raccoons from the croplands in Hokkaido, Japan

Analysis of stomach contents revealed that raccoons in the croplands of Hokkaido, Japan, were exposed to a variety of pesticides, with high concentrations of neonicotinoid insecticides like imidacloprid, acetamiprid, and clothianidin. The

concentrations of neonicotinoid insecticides in the stomach contents were found to be comparable to those identified in urine samples (chapter 1). A significant difference in pesticide concentrations was observed between raccoons that ingested corn and those that did not, underscoring the importance of corn as a substantial contributor to pesticide exposure for raccoons inhabiting croplands. While most neonicotinoids are excreted in urine as metabolites, dinotefuran's parent compound is primarily excreted in urine with few metabolites (Harada et al., 2016; Ikenaka et al., 2019). Moreover, the time to peak concentration (T_{max}) was 0.50 h after oral administration of 50 mg/kg dinotefuran to rats (Pfeil & Tasheva, 2012). Therefore, raccoons likely absorb dinotefuran rapidly, and its concentrations may decrease while raccoons were in the box trap after capture. These factors may explain the relatively high detection rate of dinotefuran in urine samples compared to that in stomach contents, especially when compared with other neonicotinoids. Additionally, a high detection rate was observed for the fungicides carbendazim and tricyclazole. Carbendazim was the most abundantly used fungicide in Japan in 2018 (Chemical Database of National Institute for Environmental Studies, Japan; https://www.nies.go.jp/kisplus/src_dvr/dvr), while tricyclazole is currently detected in large quantities in agricultural regions of Japan (Tazunoki et al., 2022; Chemical Database of National Institute for Environmental Studies, Japan; https://www.nies.go.jp/kisplus/src_dvr/dvr).

Benzene hexachloride (BHC) was the predominant organochlorine pesticide extensively used in farmlands until its ban in Japan in 1971 due to environmental persistence and animal health risks (Solomon et al., 1977). Residual BHC is likely to persist in the soil, accumulating in crops through soil-plant transfer. Dichlofenthion, an organophosphate insecticide banned in Japan since 2012, was also detected in the

raccoons. Unlike BHC, dichlofenthion has a short half-life in both the environment (243 days; Bro-Rasmussen et al., 1970) and the body (10.6 hours; Yamanaka et al., 1996). Therefore, prolonged exposure of raccoons to dichlofenthion residues is unlikely. Despite the prohibition of these substances, stockpiles may persist in personal and commercial spaces such as houses and barns, possibly serving as an alternative or additional route of exposure for raccoons in the assessed agricultural region.

4.2. Pesticides detected in masked palm civets in the metropolitan areas in Tokyo, Japan

Rice, potato, and apple were identified in the stomach contents of masked palm civets living in metropolitan areas of Tokyo; however, pesticide concentrations could not be conclusively attributed to a particular crop, raising the possibility of other routes of exposure. Thiabendazole had the highest detection rate. Masked palm civets in metropolitan areas inhabit house attics, which are generally constructed of wood (Krishnakumar et al., 2002). Thiabendazole is an antifungal and antiparasitic agent used to preserve wood (Petric, 2013). Given the high detection rates of thiabendazole in masked palm civets in metropolitan areas, daily exposure to treated wood is a very likely source of contamination.

The detection rate of carbendazim was similar in raccoons (70.7%) and masked palm civets (76.6%); however, detection rates of tricyclazole were considerably lower in masked palm civets (10%) than in raccoons (56.1%). These differences can be explained by the frequent exposure of masked palm civets in metropolitan areas to carbendazim, an industrial antifungal agent found in products like paint, textiles, paper, and leather (Singh et al., 2016). In contrast, tricyclazole is primarily used as a fungicide

in agriculture, particularly for crops like vegetables (Pareja et al., 2011). Neonicotinoid insecticides were also the most frequently detected pesticides in masked palm civets. Vegetables, fruit, and grain produced for human consumption contain neonicotinoids (Zhang et al., 2018; Craddock et al., 2019), which are likely consumed by masked palm civets when dustbins or stores are raided. Differences in the detection rates of neonicotinoids between raccoons and masked palm civets were observed. Thiacloprid was detected only in the masked palm civets, whereas thiamethoxam was detected only in the raccoons. This could be because thiacloprid can be used for apples, which were detected as a crop in the stomach contents of masked palm civets, while thiamethoxam is approved for use for corn, which was the most frequently detected crop in the stomach contents of raccoons (Chemical Database of the National Institute for Environmental Studies, Japan; https://www.nies.go.jp/kisplus/src_dvr/dvr).

4.3. Evaluation of pesticide-induced toxicity risk in wildlife

The estimated maximum exposure levels for all detected pesticides in raccoons and masked palm civets were below the ADI established for humans. The ADI is calculated by dividing observed adverse effect levels by a safety coefficient that reflects interspecific variations in chemosensitivity, highlighting substantial differences among species (Walker, 1998). There are large interspecies differences in chemosensitivity (Walker, 1998), and raccoons have a particularly low metabolic capacity for cytochrome P450, a key enzyme crucial for the detoxification of numerous pesticides (Shinya et al., 2022). The potential for synergistic interactions and additive effects, especially in scenarios of exposure to multiple pesticides, has been documented in mammals (Rizzati et al., 2016). In addition to wildlife, various pesticides have also been

detected in Japanese humans (Li & Kannan, 2018; Ichikawa et al., 2019; Oya et al., 2020). Therefore, pesticides with estimated maximum exposure levels below the ADI may have an impact on wildlife, humans and the environment. In addition, wildlife may be exposed to pesticides above the effect level in certain situations, such as immediately after pesticide application. Instances of acute toxicity in wildlife have been reported in Japan (Tahara & Nagahora, 2010; Okada & Asakawa, 2021).

This study confirmed the presence of both contemporary pesticides, such as neonicotinoids, and legacy pesticides, exemplified by BHC. Neonicotinoids have been associated with various forms of toxicity in mammals, including neurotoxicity, reproductive toxicity, hepatotoxicity, genotoxicity, and others (Thompson et al., 2020). Given BHC's extended persistence, it may remain in the body, posing prolonged toxicological risks. The potential for synergistic interactions resulting from exposure to this array of pesticides warrants consideration.

Monitoring exposure and diagnosing poisoning related to contemporary pesticides in wildlife are infrequently conducted worldwide. The method for simultaneous quantitative analysis established in this study has the potential to enhance our understanding of pesticide exposure levels. With the possibility of increased pesticide use accompanying global population growth, future research should focus on the patterns of pesticide exposure in various wildlife species and regions to contribute to wildlife conservation efforts.

5. Conclusions

In conclusion, this study sheds light on the current pesticide exposure status of wild raccoons in croplands and masked palm civets in metropolitan areas in Japan. The

results show a wide range of pesticides in both species, with notable concentrations of neonicotinoid insecticides and certain fungicides. Interestingly, raccoons showed significant exposure to pesticides associated with corn consumption, while masked palm civets showed exposure to industrial antifungal agents and neonicotinoid insecticides, probably from various urban sources. Although the estimated maximum exposure levels of the detected pesticides were below the acceptable daily intake for humans, potential synergistic effects and acute toxicity risks warrant further investigation. Overall, this study highlights the importance of monitoring and managing pesticide use to mitigate potential adverse effects on wildlife and ecosystems in transformed landscapes. Further research focusing on the long-term effects of pesticide exposure on wildlife health and ecosystem dynamics is essential for effective conservation management efforts in the region.

Table 2-1: Food type in stomach contents of raccoons and masked palm civets.

Food category	Frequency of occurrence (%)	
	Raccoons	Masked palm civets
Insects	2.4 (n=1)	6.7 (n=2)
Reptiles	2.4 (n=1)	0.0 (n=0)
Birds	0.0 (n=0)	10.0 (n=3)
Plants	92.7 (n=38)	16.7 (n=5)
Wild berries	17.1 (n=7)	6.7 (n=2)
Crops	51.2 (n=21)	40.0 (n=12)
	Corn: 51.2 (n=21)	Rice: 13.3 (n=4)
	-	Potato: 10.0 (n=3)
	-	Apple: 23.3 (n=7)

Table 2-2: Pesticide concentrations and detection rates in stomach contents of raccoons from croplands in Hokkaido

Category	Pesticide	Detection rate (%)	Concentration (Mean $\pm$ SD, ng/g)	Maximum exposure dose (μ g/kg)	Ratio to ADI ¹ (%)	ADI ² (μ g/kg/day)
Insecticide	Imidacloprid	41.5	4.36 $\pm$ 13.4	5.15	8.58	60
	Acetamiprid *	39.0	0.40 $\pm$ 0.43	0.18	0.72	25
	Clothianidin *	36.6	1.54 $\pm$ 1.60	0.43	0.44	97
	Benzene hexachloride	12.9	4.16 $\pm$ 3.00	0.40	8.00	5
	Thiamethoxam	12.2	5.89 $\pm$ 9.70	2.26	8.69	26
	Permethrin	6.45	67.7 $\pm$ 23.4	8.20	16.4	50
	Diazinon	2.44	8.71	0.78	78.0	1
	Diclofenthion	2.44	5.10	0.46	18.4	2.5
	Chlorpyrifos-methyl	2.44	0.95	0.09	0.09	10
	Pirimiphos-methyl	2.44	1.99	0.18	4.50	4
	Fenitrothion	2.44	3.05	0.27	5.40	5
	Chlorpyrifos	2.44	7.43	0.67	67.0	1
	Dinotefuran	2.44	0.34	0.03	0.01	220
Herbicide	Diflufenican	12.9	8.16 $\pm$ 6.91	1.80	0.90	200
	Benzofenap	9.76	2.99 $\pm$ 0.57	0.35	17.50	2
	Trifluralin	9.68	82.9 $\pm$ 113	21.9	91.3	24
	Linuron	4.88	0.80 $\pm$ 0.01	0.07	2.33	3
	Benzobicyclon	2.44	0.46	0.04	0.12	34
	Halosulfuran-methyl	2.44	0.31	0.03	0.05	63
Fungicide	Carbendazim	70.7	1.64 $\pm$ 2.67	1.39	6.95	20
	Tricyclazole *	56.1	2.59 $\pm$ 5.22	2.14	7.13	30
	Tebuconazole	16.1	8.59 $\pm$ 3.97	1.34	4.47	30
	Flutolanil	14.6	0.39 $\pm$ 0.39	0.05	0.06	90
	Metalaxyl	9.76	1.13 $\pm$ 1.09	0.27	0.34	80
	Thiabendazole	7.32	0.19 $\pm$ 0.12	0.03	0.03	100
	Azoxystrobin	4.88	0.55 $\pm$ 0.05	0.05	0.03	200
	Diuron	4.88	0.20 $\pm$ 0.01	0.02	0.29	7
	Isoprothiolane	4.88	85.6 $\pm$ 84.6	15.3	15.3	100
	Propiconazole	4.88	4.23 $\pm$ 2.01	0.56	1.40	40
	Metconazole	2.44	1.11	0.10	1.00	10

* Significantly higher concentrations were detected in raccoons with confirmed crops in the stomach contents than those in raccoons without confirmed crops in the stomach contents ($p < 0.05$).

¹ Ratio to ADI: calculated by dividing the estimated maximum exposure dose by ADI.

² ADI: acceptable daily intake; obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>)

**Table 2-3: Detection rates of neonicotinoid insecticides
in stomach contents and urine of raccoons (%)**

Neonicotinoid	Stomach content (in this study)	Urine (in chapter 1)
Imidacloprid	41.5	23.4
Acetamiprid	39.0	35.6
Clothianidin	36.6	66.1
Thiamethoxam	12.2	18.6
Dinotefuran	2.44	20.3
Thiacloprid	0	3.39
Nitenpyram	0	0

Table 2-4: Pesticide concentration and detection rates in stomach contents of masked palm civets from the metropolitan area in Tokyo

Category	Pesticide	Detection rate (%)	Concentration (Mean $\pm$ SD, ng/g)	Estimated maximum exposure dose (μ g/kg)	Ratio to ADI ¹ (%)	ADI ² (μ g/kg)
Insecticide	Acetamiprid	43.3	1.18 $\pm$ 2.28	0.64	2.56	25
	Thiacloprid	33.3	1.14 $\pm$ 0.46	0.22	2.20	10
	Clothianidin	10.0	4.01 $\pm$ 3.02	0.67	0.69	97
	Imidacloprid	6.67	1.11 $\pm$ 6.65	0.11	0.18	60
Fungicide	Carbendazim	76.7	1.72 $\pm$ 1.86	0.79	3.95	20
	Thiabendazole	70.0	0.93 $\pm$ 1.54	0.70	0.70	100
	Tricyclazole	10.0	0.92 $\pm$ 0.02	0.09	0.30	30
	Diuron	6.67	1.11 $\pm$ 0.10	0.11	1.57	7
	Metalaxyl	6.67	1.67 $\pm$ 1.18	0.28	0.35	80
	Boscalid	3.33	29.0	2.61	6.53	40

¹ Ratio to ADI: calculated by dividing the estimated maximum exposure dose by ADI.

² ADI: acceptable daily intake; obtained from the PubChem database (<https://pubchem.ncbi.nlm.nih.gov/>)

Supplementary data

Table S2-1. Information of raccoons used in this study.

Table S2-2. Information of masked palm civets used in this study.

Table S2-3. Measurement conditions of LC/MS/MS.

Table S2-4. Measurement conditions of GC/MS/MS.

Table S2-1. Information of raccoons used in this study.

Date of capture	Sex	Body weight (kg)	Age
Mar/18/2020	♀	5.30	Adult
Aug/06/2020	♂	2.78	Cub
May/26/2021	♂	6.70	Adult
Jun/14/2021	♂	5.48	Adult
Jun/19/2021	♀	5.64	Adult
Jul/01/2021	♂	4.70	Adult
Sep/09/2021	♂	4.50	Cub
Sep/10/2021	♂	4.56	Cub
Sep/10/2021	♀	5.02	Adult
Nov/30/2021	♂	9.62	Adult
Feb/28/2022	♀	4.40	Adult
Mar/24/2022	♂	6.56	Adult
Mar/25/2022	♂	5.24	Adult
Mar/28/2022	♀	4.26	Adult
Mar/31/2022	♂	6.44	Adult
Mar/31/2022	♀	2.36	Cub
Apr/13/2022	♂	4.64	Adult
Apr/13/2022	♀	3.84	Adult
Apr/13/2022	♀	4.72	Adult
Apr/26/2022	♂	6.16	Adult
May/01/2022	♀	4.94	Adult
May/03/2022	♂	3.22	Cub
May/05/2022	♂	7.40	Adult
May/05/2022	♂	6.32	Adult
May/07/2022	♂	5.48	Cub
May/08/2022	♂	4.02	Cub
May/10/2022	♂	5.96	Adult
May/28/2022	♀	7.18	Adult
May/20/2022	♂	7.12	Adult
May/23/2022	♀	5.76	Adult
May/23/2023	♀	4.67	Adult
May/23/2022	♂	6.68	Adult

May/25/2022	♂	7.56	Adult
May/27/2022	♂	5.50	Cub
May/30/2022	♂	5.40	Adult
May/31/2022	♀	4.46	Adult
Jun/03/2022	♂	5.62	Adult
Jun/09/2022	♀	5.18	Adult
Jun/09/2022	♀	6.32	Adult
Jun/22/2022	♂	5.94	Adult
Jul/06/2022	♀	6.48	Adult

Table S2-2. Information of masked palm civets used in this study.

Date of capture	Sex	Body weight (kg)	Age
Sep/06/2021	♂	1.4	Cub
Sep/13/2021	♀	1.5	Cub
Sep/22/2021	♀	4.1	Adult
Oct/13/2021	♂	0.5	Cub
Oct/13/2021	♀	0.5	Cub
Oct/13/2021	♀	0.4	Cub
Oct/13/2021	♀	0.4	Cub
Oct/13/2021	♀	0.4	Cub
Oct/16/2021	♀	3.4	Adult
Oct/22/2021	♀	4.0	Adult
Oct/23/2021	♀	4.6	Adult
Dec/04/2021	♀	4.3	Adult
Mar/22/2022	♂	4.0	Adult
Mar/24/2022	♂	3.2	Adult
Apr/01/2022	♂	3.4	Adult
Apr/05/2022	♂	3.5	Adult
Apr/13/2022	♂	2.6	Cub
Apr/23/2022	♀	0.7	Cub
Apr/25/2022	♀	1.8	Cub
Apr/28/2022	♀	4.3	Adult
May/21/2022	♀	2.2	Cub
May/26/2022	♂	1.5	Cub
May/5/2022	♂	1.6	Cub
May/5/2022	♀	4.2	Adult
Jun/18/2022	♀	3.2	Adult
Jun/24/2022	♂	2.3	Cub
Jul/08/2022	♂	1.6	Cub
Aug/03/2022	♀	1.5	Cub
Sep/07/2022	♀	2.2	Cub
Sep/12/2022	♂	2.5	Cub

Table S2-3. Measurement conditions of LC/MS/MS.

	Category	Retention time (min)	Recovery rate (%)	PQL (ng/g)	Polarity	Precursor ion	Product ion	Collision energy (eV)
Abamectin	I	27.71	106.50	2.46	+	890.5	567.3 305.1	8 20
Acephate	I	4.44	87.01	0.11	+	184.0	143.0 95.0	10 20
Acetamiprid	I	11.81	102.53	2.26	+	223.08	126.0 56.3	24 16
Acetamiprid-d3	Internal STD	11.76	107.65	-	+	226.69	126.2 59.2	24 16
Acibenzola-methyl	F	19.89	104.93	0.50	+	211.0	136.0 91.0	32 20
Acifluorfen	H	18.67	85.59	0.34	-	360.0	316.0 194.9	10 20
Aldicarb	I	13.90	106.74	0.16	+	116	89.0 70.0	4 4
Aldicarb-sulfoxide	I	6.43	114.23	0.19	+	207.1	132.0 89.1	10 8
Aldoxycarb	I	7.08	71.57	0.30	+	240.0	223.0 148.0	10 8
Ametryn	H	20.14	72.52	0.29	+	228.1	186.0 68.0	16 40
Aminocarb	I	16.12	111.07	0.22	+	209.1	152.2 137.2	12 24
Anilofos	H	23.15	100.60	0.84	+	368.0	198.9 124.9	8 32

Azamethiphos	I	15.64	88.31	0.53	+	325.0	182.9 111.9	12 40
Azimsulfuron	H	10.81	135.59	0.12	+	425.1	182.0 83.0	12 40
Azinphosmethyl	I	19.48	71.26	0.18	+	318.0	261.0 125.0	10 12
Azoxystrobin	F	20.21	103.62	0.31	+	404.0	372.0 344.0	10 20
Benalaxyl	F	25.20	100.32	2.93	+	326.2	148.1 91.0	16 40
Bendiocarb	I	16.07	80.06	0.19	+	224.1	167.0 109.0	4 14
Bensulfuron-methyl	H	17.54	107.64	0.28	+	411.1	182.0 149.0	16 16
Bentazone	H	8.48	89.79	0.34	-	239.0	197.0 132.0	20 25
Benzobicyclon	H	21.16	89.77	1.03	+	447.1	257.1 229.0	20 36
Benzofenap	H	24.81	85.97	1.20	+	431.1	119.0 105.0	20 36
Boscalid	F	20.68	80.85	5.86	+	343.0	307.0 271.1	16 36
Bromuconazole	F	23.72	99.19	0.03	+	376.0	158.9 70.1	28 20
Butafenacil	H	21.82	73.76	0.08	+	492.1	331.0 179.9	20 40
Butamifos	H	23.73	126.86	0.50	+	333.1	180.0 152.0	5 10
Cafenstrole	H	21.42	67.53	0.38	+	351.2	100.0	4

							72.1	24
Carbaryl	I	16.86	94.03	0.20	+	202.0	145.0 127.0	5 20
Carbendazim	F	12.87	126.18	0.61	+	192.1	160.0 132.1	12 32
Carbofuran	I	16.16	137.91	0.33	+	222.1	165.0 123.0	8 20
Carpropamid	F	23.16	91.84	0.14	+	334.1	196.0 138.9	8 20
Chlorfenvinphos	I	23.64	138.95	0.33	+	359.0	170.0 155.1	40 8
Chlorfluazuron	IGR	26.69	103.71	0.92	+	540.0	382.9 158.0	20 16
Chloridazon	H	11.77	82.86	0.24	+	222.0	104.0 77.0	24 36
Chlorimuron-ethyl	H	15.37	103.78	0.16	+	415.1	186.0 83.0	12 40
Chlorobenzilate	I	23.25	117.94	1.61	+	342.1	179.0 149.0	15 20
Chloroxuron	H	21.42	119.56	0.07	+	291.1	72.0 46.1	20 20
Chlorsulfuron	H	11.15	90.98	0.25	+	358.0	167.0 141.1	20 20
Cinosulfuron	H	11.62	107.16	0.25	+	414.1	183.0 83.0	12 40
Clodinafop acid	H	16.49	91.04	0.21	+	312.1	266.0 91.0	12 28
Clofencet	PGR	11.36	77.72	0.22	+	279.1	261.1 166.6	15 30

Clofentezine	IGR	23.61	121.12	0.45	+	303.0	138.0 102.0	12 40
Clomeprop	H	25.19	102.49	0.25	+	324.1	202.9 120.0	12 24
Cloquintocet-mexyl	H	25.52	73.57	0.46	+	336.1	238.0 192.0	12 28
Cloransulam-methyl	H	15.28	82.63	0.10	+	430.0	398.0 369.9	8 20
Clothianidin	I	10.60	137.26	0.36	+	250.0	169.0 132.0	12 16
Clothianidin-d3	Internal STD	10.57	130.72	-	+	253.0	171.9 132.0	8 16
Coumaphos	I	23.33	73.41	0.34	+	363.0	306.9 227.0	16 24
Cumyluron	H	12.40	67.96	0.19	+	303.1	185.0 124.9	8 36
Cyanophos	I	21.76	96.21	0.89	+	244.0	202.2 160.2	20 24
Cyazofamid	F	22.11	75.18	0.13	+	325.1	261.0 107.9	8 8
Cyclanilide	PGR	16.53	121.83	0.25	-	272.0	228.0 159.9	10 12
Cycloate	H	24.92	121.35	0.98	+	216.1	83.0 55.1	12 32
Cyclosulfamuron	H	18.65	74.54	0.19	+	422.1	261.0 218.0	12 24
Cyflufenamid	F	23.74	104.63	0.79	+	413.1	295.1 241.0	12 20
Diclosulam	H	15.58	76.91	0.18	+	406.0	378.0	10

							161.0	40
Diflubenzuron	I	22.36	79.33	0.13	+	311.0	158.0 141.0	8 32
Dinotefuran	I	6.47	87.46	0.12	+	203.1	157.0 129.0	10 12
Dinotefuran-d3	Internal STD	6.44	94.45	-	+	206.1	132.1 90.0	12 20
Dioxacarb	I	11.53	77.56	0.12	+	224.0	167.0 123.0	10 10
Diuron	H	18.69	68.88	0.21	+	235.0	72.0 72.1	20 20
Ethoxysulfuron	H	15.70	124.86	0.21	+	399.1	261.0 218.0	10 25
Fenamidone	F	20.45	108.12	0.12	+	312.0	92.2 65.1	28 56
Fenoxycarb	IGR	22.56	123.35	0.31	+	302.1	116.0 88.0	17 29
Flazasulfuron	H	12.16	95.82	0.20	+	408.1	182.1 139.1	28 40
Fluacrypyrim	I	24.27	107.70	0.66	+	427.1	205.1 145.1	10 20
Fluridone	H	19.82	80.04	0.40	+	330.1	310.0 309.0	28 40
Fluroxypyr	H	8.76	130.22	0.28	+	255.0	208.9 181.0	16 24
Flutolanil	F	20.94	122.85	0.43	+	324.1	262.0 242.0	16 24
Fomesafen	H	18.71	121.91	0.19	+	456.0	344.0 223.0	8 36

Foramsulfuron	H	12.36	95.65	0.15	+	453.1	272.0 182.0	8 16
Forchlorfenuron	PGR	18.87	80.84	0.08	+	248.1	129.0 93.0	16 36
Furametpyr	F	18.37	79.31	0.48	+	334.1	290.1 156.9	12 32
Furathiocarb	I	25.24	92.15	0.89	+	383.2	252.0 195.0	8 12
Halosulfuron-methyl	H	14.12	98.62	0.18	+	435.1	182.0 83.0	16 40
Haloxyfop	H	19.29	102.49	1.54	+	362.0	316.0 91.0	16 32
Hexaflumuron	I	24.50	93.72	0.38	+	461.0	158.0 140.9	20 32
Hexythiazox	I	25.91	108.52	6.18	+	353.0	228.0 168.0	11 23
Imazalil	F	23.09	79.31	0.17	+	297.1	158.9 69.0	20 16
Imazaquin	H	10.62	140.55	0.09	+	312.1	266.7 86.0	20 28
Imazosulfuron	H	12.41	77.01	0.08	+	413.1	156.0 152.9	16 8
Imibenconazole	F	25.45	92.55	0.59	+	411.0	171.0 125.0	16 32
Imibenconazole-dibenzyl	F	15.97	113.06	0.05	+	271.0	173.9 70.2	24 20
Imidacloprid	I	10.56	94.96	0.30	+	256.1	209.0 175.0	12 16
Inabenfide	H	20.37	118.27	1.44	+	339.1	321.1	20

							80.2	32
Indanofan	H	22.06	119.22	2.34	+	341.1	187.0 175.0	8 4
Indoxacarb	I	24.37	63.63	0.25	+	528.1	249.0 150.0	12 20
Iodosulfuron-methyl	H	14.13	132.73	0.07	+	508.0	167.0 56.1	16 40
Iprobenfos	I	22.83	78.52	0.75	+	289.1	204.9 91.0	4 16
Isazophos	I	21.49	98.55	0.48	+	314.1	162.0 120.0	10 28
Isoprocarb	I	18.14	82.24	0.48	+	194.1	137.0 95.0	4 8
Isoprothiolane	F	20.98	134.19	0.45	+	291.1	231.0 188.9	4 16
Lactofen	H	25.27	150.96	0.70	+	479.1	344.0 300.0	8 28
Linuron	H	19.99	70.87	0.14	+	249.0	182.0 159.9	12 12
Lufenuron	IGR	25.58	71.91	0.30	+	511.0	158.0 140.9	12 40
Mandipropamid	F	20.85	81.57	0.19	+	412.1	356.1 328.1	4 8
Mefenacet	H	21.41	68.07	0.35	+	299.1	148.0 120.0	8 24
Mepanipyrim	F	21.60	91.86	0.34	+	224.1	106.0 77.0	24 40
Mesosulfuron-methyl	H	13.55	104.18	0.13	+	504.1	182.0 83.0	20 40

Metalaxyl	F	18.78	115.97	0.58	+	208.2	248.0 220.1	5 8
Metconazole	F	23.62	126.95	0.06	+	320.2	125.0 70.1	40 20
Methabenzthiazuron	H	18.09	78.57	0.19	+	222.1	165.0 150.0	12 36
Methidathion	I	19.14	78.09	2.31	+	303.0	145.0 85.0	4 16
Methiocarb	I	20.29	61.86	0.15	+	226.1	169.0 121.0	4 16
Methomyl	I	8.05	114.87	0.10	+	163.1	106.0 88.0	4 4
Methoxyfenozide	I	21.18	78.61	0.20	+	369.2	313.1 149.0	10 12
Metolcarb	I	14.76	81.49	0.21	+	166.1	109.1 91.0	5 20
Metosulam	H	14.88	97.67	0.17	+	418.0	174.9 139.9	24 40
Metsulfuron-methyl	H	10.19	66.32	0.16	+	382.1	198.9 167.0	10 12
Monolinuron	H	17.09	129.71	0.36	+	215.1	148.0 125.9	12 16
Naproanilide	H	22.45	84.66	0.50	+	292.1	171.0 120.0	8 28
Napropamide	H	22.00	150.50	0.26	+	272.2	171.0 58.1	16 28
Naptalam	H	13.45	76.99	0.12	+	292.1	149.0 144.0	20 4
Nitenpyram	I	7.54	106.83	0.46	+	271.1	225.1	4

							56.1	36
Nitenpyram-d3	Internal STD	7.52	111.11	-	+	274.1	228.2	8
							126.0	24
Oryzalin	H	22.05	74.20	3.27	+	345.1	281.1	12
							161.1	24
Oxamyl	I	7.50	74.12	0.19	+	237.1	90.0	10
							72.0	12
Pirimicarb	I	17.97	102.66	0.32	+	239.2	182.1	12
							72.1	20
Prochloraz	F	23.71	103.21	0.06	+	376.0	308.0	4
							265.9	12
Promecarb	I	20.69	109.54	0.06	+	208.1	151.0	10
							109.1	10
Propiconazole	F	23.37	73.59	0.46	+	342.1	159.0	32
							69.1	16
Propoxur	I	15.93	127.78	0.31	+	210.1	168.1	10
							153.1	10
Quizalofop-ethyl	H	24.99	109.39	0.24	+	373.1	271.2	24
							255.1	36
Spirodiclofen	I	26.73	138.23	1.08	+	411.1	313.0	5
							71.2	15
Sulfosulfuron	H	12.16	115.93	0.12	+	471.1	218.1	24
							211.1	8
Teflubenzuron	I	25.38	69.16	0.14	-	379.0	359.0	10
							339.0	4
Thiabendazole	F	14.65	133.90	0.23	+	202.0	175.0	24
							131.0	36
Thiacloprid	I	13.09	100.11	0.09	+	253.0	125.9	20
							90.0	40

Thiacloprid-d4	Internal STD	13.05	92.69	-	+	257.1	126.0 90.1	28 50
Thiamethoxam	I	8.61	73.48	0.37	+	292.0	211.0 181.0	16 24
Thiamethoxam-d4	Internal STD	8.57	97.30	-	+	296.1	215.1 185.2	8 24
Thifensulfuron-methyl	H	10.18	127.89	0.11	+	388.0	167.0 56.0	12 40
Thiobencarb	H	23.87	102.16	0.19	+	258.1	125.0 100.1	16 8
Thiocyclam	I	15.04	95.45	0.19	+	182.0	136.9 73.0	12 24
Thiodicarb	I	17.77	109.40	0.21	+	355.1	107.9 88.0	8 12
Tiadinil	F	21.32	71.51	0.37	-	266.0	238.0 71.1	4 12
Tolyfluanid	F	24.94	131.88	0.40	+	364.0	290.0 119.0	16 28
Tralkoxydim	H	20.49	127.65	0.28	+	330.2	284.1 138.0	8 16
Triadimefon	H	21.19	121.65	0.20	+	294.1	197.0 69.0	10 20
Triasulfuron	H	12.51	104.30	0.12	+	402.1	167.0 141.0	12 16
Triazophos	I	21.49	98.55	0.48	+	314.1	162.0 97.0	16 40
Tribenuron-methyl	H	12.80	91.80	0.20	+	396.1	181.0 155.0	16 8
Trichlorfon	I	11.18	80.12	0.05	+	256.9	221.0	4

							109.0	12
Tricyclazole	F	13.74	100.98	0.75	+	190.0	163.0	25
							136.0	25
Trifloxystrobin	F	24.44	104.85	0.27	+	409.1	206.0	8
							186.0	12
Trifloxysulfuron	H	14.36	72.03	0.21	+	438.1	182.0	16
							83.0	40
Triflumizole	F	24.61	82.38	0.19	+	346.1	278.0	4
							43.1	24
Triflumuron	IGR	23.69	107.90	0.08	+	359.0	155.9	12
							138.9	32
Triflusulfuron-methyl	H	17.14	129.54	0.36	+	493.1	264.0	20
							96.0	44
Uniconazole P	PGR	8.60	134.36	0.18	+	292.0	125.0	28
							70.0	25
Vamidothion	I	11.77	72.71	0.13	+	288.1	146.0	4
							58.1	40
XMC	I	17.00	75.36	0.41	+	180.1	123.0	4
							95.1	20
Xylylcarb	I	17.34	71.28	0.56	+	180.1	123.0	4
							108.0	28
Zoxamide	F	18.39	89.34	0.30	+	336.0	186.9	20
							158.9	40

I: insecticide, H: herbicide, F: fungicide, IGR: insect growth regulator, PGR: plant growth regulator

Table S2-4. Measurement conditions of LC/MS/MS.

	Category	Retention time (min)	Recovery rate (%)	PQL (ng/g)	Quantification		Confirmation	
					MRM (<i>m/z</i>)	Collision energy (eV)	MRM (<i>m/z</i>)	Collision energy (eV)
1,1-dichloro-2,2-bis(4-ethylphenyl)ethane	I	15.88	117.3 113.4	0.22	223.2 > 167.1	14	223.2 > 179.1 223.2 > 193.1	22 28
2,6-dichlorobenzamide	H	10.21	76.2 72.5	0.66	172.90>145.00	18	188.9 > 172.9 172.9 > 109.0	8 26
2-1-naphthylacetamide	PGR	13.06	127.6 99.7	0.47	141.1 > 115.1	18	185.1 > 141.1 141.1 > 91.0	22 24
Acephate-d6 (ISTD)	Internal STD	7.59	105.3 103.2	-	139.1 > 42.0	10	139.1 > 97.0	14
Alachlor	H	12.64	87.2 119.8	0.40	188.1 > 160.1	10	188.1 > 132.1 160.1 > 132.1	18 10
Allethrin	I	19.77	134.6 104.5	0.48	123.1 > 81.1	10	136.1 > 93.1 123.1 > 95.1	14 8
Allidochlor	H	6.28	117.7 111.0	0.72	132.1 > 56.0	8	138.1 > 96.0 132.1 > 49.0	6 24
Ametryn	H	12.79	80.6 98.8	0.69	227.1 > 185.1	6	227.1 > 58.0 227.1 > 170.1	14 14
Amitraz	I	18.77	93.9 104.7	0.21	162.1 > 132.1	10	293.1 > 162.1 162.1 > 106.1	8 24
Anilofos	H	18.10	71.3 108.0	0.56	226.1 > 157.0	14	226.1 > 184.0 184.0 > 157.0	6 10
Atrazine	H	11.12	102.6 91.3	0.60	215.10>58.00	14	215.1 > 173.1 200.1 > 104.1	6 18
Azaconazole	F	15.61	110.7	0.92	216.9 > 172.9	14	172.9 > 145.0	16

			128.5				216.9 > 145.0	26
Azinphos-ethyl	I	19.11	138.0	0.80	132.1 > 77.0	6	160.1 > 132.1	20
			85.5				160.1 > 77.0	14
Azinphos-methyl	I	18.52	132.7	0.84	160.1 > 132.1	6	160.1 > 77.0	20
			109.6				132.1 > 77.0	14
Azoxystrobin	F	22.29	74.6	0.55	344.1 > 329.1	16	388.1 > 345.1	18
			110.4				388.1 > 300.0	20
Benalaxyl	F	16.63	76.3	0.19	148.1 > 105.1	16	148.1 > 79.1	24
			72.7				148.1 > 133.1	14
Benfluralin	H	10.27	86.7	0.33	292.1 > 264.0	8	292.1 > 160.0	22
			92.1				292.1 > 206.0	12
Benfuresate	H	12.33	102.1	0.91	256.1 > 163.1	10	163.1 > 121.1	6
			103.3				163.1 > 91.0	22
Benoxacor	H	12.13	93.0	0.55	176.0 > 120.0	10	259.0 > 120.0	18
			117.9				259.0 > 176.0	8
BHC (four isomers)	I	10.63 / 11.13 / 11.32 / 11.87	81.1	0.51	180.9 > 144.9	16	218.9 > 182.9	8
			103.7				218.9 > 144.9	20
Bifenazate	I	17.89	126.2	0.43	300.1 > 258.1	8	258.1 > 196.1	12
			101.0				300.1 > 199.1	20
Bifenox	H	18.13	103.8	0.47	340.9 > 309.9	10	340.9 > 188.9	20
			131.3				309.9 > 188.9	12
Bifenthrin	I	17.80	90.4	0.67	181.1 > 166.1	12	181.1 > 179.1	12
			108.4				181.1 > 153.1	8
Bitertanol (two isomers)	F	19.53 / 19.64	84.0	0.69	170.1 > 141.1	22	170.1 > 115.1	28
			98.0				141.1 > 115.1	18
Bromobutide	H	12.49	105.8	0.74	119.1 > 91.0	10	119.1 > 65.0	26
			109.1				232.2 > 176.1	10
Bromuconazole (two isomers)	F	17.73 / 18.15	85.6	0.40	172.9 > 145.0	18	172.9 > 109.0	26
			77.8				294.9 > 172.9	14

Bupirimate	F	15.52	87.2 137.2	0.91	273.1 > 108.1	16	273.1 > 193.1 316.1 > 208.1	8 10
Buprofezin	I	15.53	114.1 73.4	0.22	172.1 > 57.0	14	175.1 > 132.1 175.1 > 117.1	12 24
Butafenacil	H	19.90	90.6 72.5	0.70	331.0 > 180.0	14	180.0 > 124.0 180.0 > 152.0	18 10
Cafenstrole	H	20.05	131.8 80.2	0.54	100.1 > 72.0	6	188.1 > 119.1 188.1 > 82.0	20 18
Captan	F	20.19	110.3 115.3	0.61	149.1 > 79.1	14	149.1 > 105.1 149.1 > 70.0	4 18
Carbaryl-d7 (ISTD)	Internal STD	12.71	129.7 136.1	-	151.1 > 121.1	18	151.1 > 123.1	16
Carbofuran	I	10.99	98.5 93.8	0.14	164.1 > 149.1	8	149.1 > 121.1 164.1 > 103.1	10 24
Carbophenothion	I	16.69	81.5 127.5	0.91	157.0 > 45.0	18	341.9 > 157.0 341.9 > 199.0	14 8
Carboxin	F	15.56	74.4 81.6	0.89	143.0 > 87.0	8	235.1 > 143.0 235.1 > 87.0	12 24
Carfentrazone-ethyl	H	16.62	99.9 88.7	0.81	340.1 > 312.1	14	312.1 > 151.1 340.1 > 151.1	24 28
Chlomethoxynil	H	17.59	89.4 70.2	0.85	312.9 > 265.9	12	265.9 > 188.9 312.9 > 282.9	16 6
Chlorbenside	I	21.35	125.8 73.1	0.95	125.0 > 89.0	16	125.0 > 99.0 127.0 > 89.0	18 18
Chlorethoxyfos	I	9.69	98.9 91.2	0.31	153.0 > 97.0	12	153.0 > 125.0 298.9 > 97.0	4 22
Chloridazon	H	16.89	110.2 103.5	0.70	221.0 > 77.0	24	223.0 > 77.0 221.0 > 105.0	24 12
Chlormephos	I	7.76	99.8	0.24	154.0 > 121.0	10	233.9 > 121.0	6

			84.6				154.0 > 65.0	4
Chlornitrofen	H	16.66	96.3	0.53	316.9 > 286.9	14	318.9 > 288.9	14
			68.6				316.9 > 196.0	24
Chlorobenzilate	I	16.02	104.6	0.34	139.0 > 111.0	16	251.0 > 139.0	14
			98.5				139.0 > 75.0	26
Chloroneb	F	8.48	75.2	0.35	206.0 > 191.0	12	206.0 > 141.0	20
			136.1				193.0 > 113.0	18
Chlorpropham	H	10.14	137.2	0.36	127.1 > 65.0	22	213.1 > 171.1	6
			121.5				127.1 > 92.0	18
Chlorpyrifos	I	13.44	70.3	0.68	196.9 > 168.9	14	313.9 > 257.9	14
			125.7				313.9 > 285.9	8
Chlorpyrifos-d10 (ISTD)	Internal STD	13.35	118.1	-	324.1 > 259.9	16	324.1 > 194.9	30
Chlorpyrifos-methyl	I	12.49	102.0	0.91	285.9 > 93.0	22	287.9 > 93.0	22
			137.1				285.9 > 270.9	14
Chlorthal-dimethyl	H	13.54	81.3	0.46	298.9 > 220.9	24	300.9 > 222.9	26
			101.3				300.9 > 272.9	14
Chlorthiophos	I	16.24	118.4	0.95	324.9 > 268.9	14	268.9 > 205.0	18
			107.3				268.9 > 177.0	24
Chlorthiophos	I	16.24	89.5	0.28	324.9 > 268.9	14	268.9 > 205.0	18
			132.2				324.9 > 296.9	8
Cinidon-ethyl	H	23.35	107.2	0.97	358.1 > 330.1	14	330.1 > 302.1	18
			120.6				330.1 > 107.0	18
Clomazone	H	11.19	79.4	0.88	204.1 > 107.0	20	204.1 > 78.0	26
			89.9				204.1 > 68.0	24
Cyanofenphos	I	16.71	102.8	0.21	185.0 > 157.0	8	303.1 > 141.0	14
			113.2				303.1 > 169.0	4
Cyflufenamid	F	15.71	119.5	0.43	412.1 > 295.1	8	294.1 > 237.1	6
			87.6				294.1 > 223.1	14

Cyhalofop-butyl	H	18.65	108.1 94.6	0.33	256.1 > 120.1	12	357.1 > 256.1 256.1 > 91.0	10 26
Cyhalothrin (two isomers)	I	18.60 / 18.78	109.3 75.5	0.44	208.0 > 181.0	8	197.0 > 141.0 197.0 > 161.0	12 8
Deltamethrin (two isomers)	I	21.91 / 22.13	122.2 85.3	0.47	180.9 > 151.9	22	252.9 > 93.0 252.9 > 171.9	20 8
Dialifos	I	19.17	90.5 70.8	0.28	208.0 > 181.0	8	208.0 > 89.0 208.0 > 130.0	24 22
Diallate (two primers)	H	10.53 / 10.71	102.0 105.7	0.88	234.1 > 150.0	20	234.1 > 192.1 128.0 > 86.0	14 4
Diazinon	I	11.54	71.3 89.3	0.17	304.1 > 179.1	10	179.1 > 137.1 179.1 > 122.1	18 24
Dichlobenil	H	6.81	114.1 82.7	0.28	170.9 > 136.0	14	170.9 > 100.0 170.9 > 110.0	24 14
Dichlofenthion	I	12.37	123.4 96.2	0.75	279.0 > 222.9	14	222.9 > 204.9 279.0 > 250.9	14 8
Dichlofluanid	F	13.25 / 13.46	111.0 108.5	0.13	223.9 > 123.1	8	167.1 > 124.1 167.1 > 97.0	10 22
Dichlorvos	I	5.90	114.3 78.3	0.25	109.0 > 79.0	8	185.0 > 93.0 185.0 > 109.0	14 14
Dichlorvos-d6	Internal STD	5.87	128.5 76.8	-	191.1 > 99.0	16	191.1 > 115.0	16
Diclobutrazol	F	15.59	92.1 73.5	0.68	270.0 > 159.0	14	272.0 > 161.0 270.0 > 201.0	14 8
Diclofop-methyl	H	17.17	99.0 116.9	0.37	340.0 > 253.0	14	253.0 > 162.0 253.0 > 145.0	22 24
Dicloran	F	10.89	131.1 88.8	0.81	206.0 > 176.0	10	176.0 > 148.0 206.0 > 124.0	12 24
Diethofencarb-d7 (ISTD)	Internal	13.41	68.9	-	274.2 > 226.1	8	274.2 > 198.1	12

	STD		89.8					
Difenoconazole (two isomers)	F	21.81 / 21.88	84.0 132.4	0.25	323.0 > 265.0	14	265.0 > 202.0 265.0 > 139.0	20 30
Diiflufenican	H	17.20	126.1 88.3	0.37	394.1 > 266.0	14	266.0 > 246.0 266.0 > 218.0	14 24
Dimethenamid	H	12.38	80.8 102.6	0.81	230.0 > 154.1	10	203.0 > 126.1 203.0 > 154.1	22 12
Dimethomorph (two isomers)	F	22.45 / 22.82	80.0 89.9	0.41	301.1 > 165.1	14	387.1 > 301.1 301.1 > 139.0	12 14
Dimethylvinphos (two isomers)	I	13.19	119.6 117.8	0.31	294.9 > 109.0	14	296.9 > 109.0 294.9 > 279.9	20 16
Diniconazole	F	16.11	105.3 123.2	0.96	268.0 > 232.0	12	270.0 > 234.0 268.0 > 149.0	10 24
Dioxabenzofos	I	10.28	106.7 100.8	0.92	216.0 > 201.0	10	216.0 > 138.0 216.0 > 183.0	10 10
Dioxathion	I	19.76	77.4 119.9	0.61	185.0 > 129.0	12	271.0 > 153.0 271.0 > 97.0	8 22
Diphenyl	F	7.26	117.9 92.4	0.17	154.1 > 128.1	22	154.1 > 115.1 152.1 > 126.1	24 24
Disulfoton	I	11.75	71.2 87.1	0.12	153.0 > 97.0	10	153.0 > 125.0 186.0 > 153.0	6 6
Dithiopyr	H	12.91	113.4	0.61	354.1 > 306.1	8	354.1 > 286.1 306.1 > 286.1	14 10
Edifenphos	F	16.74	121.4 113.6	0.78	173.0 > 109.0	10	310.0 > 173.0 310.0 > 109.0	14 26
Endosulfan	I	16.11	97.8 132.0	0.18	194.9 > 160.0	8	194.9 > 125.0 194.9 > 123.0	24 22
EPN	I	17.81	68.8 74.5	0.48	169.1 > 140.9	8	156.9 > 77.0 169.1 > 77.0	24 22

EPN-d5	Internal STD	17.78	138.5 97.9	-	174.1 > 142.0	8	174.1 > 77.0	26
Epoxiconazole	F	17.42	73.7 103.6	0.20	192.0 > 138.0	14	192.0 > 111.0 192.0 > 165.0	26 8
EPTC	H	6.92	93.3 71.3	0.69	189.1 > 128.1	4	189.1 > 86.0 189.1 > 160.1	12 8
Esprocarb	H	13.28	71.0 82.7	0.46	222.1 > 91.0	14	162.1 > 91.0 222.1 > 162.1	10 8
Ethion	I	16.18	111.9 126.1	0.16	153.0 > 97.0	14	230.9 > 129.0 153.0 > 125.0	24 6
Ethofumesate	H	13.15	121.3 71.9	0.83	207.1 > 161.1	8	207.1 > 137.1 207.1 > 179.1	12 6
Ethoprophos	I	9.89	131.0 94.5	0.54	200.0 > 158.0	6	158.0 > 97.0 158.0 > 114.0	18 8
Etobenzanid	H	20.05	83.6 95.0	0.13	179.1 > 149.0	6	179.1 > 59.0 179.1 > 121.0	10 14
Etofenprox	I	20.78	80.9 131.5	0.46	163.1 > 135.1	10	163.1 > 107.1 135.1 > 107.1	18 10
Etofenprox-d5 (ISTD)	Internal STD	20.75	126.9 88.9	-	168.2 > 136.1	10	168.2 > 108.1	16
Etoxazole	I	17.95	105.3 90.5	0.49	359.1 > 187.1	14	330.1 > 300.1 359.1 > 340.1	26 12
Etridiazole	F	7.89	118.1 90.4	0.80	210.9 > 182.9	10	182.9 > 139.9 210.9 > 139.9	18 22
Etrimfos	I	11.87	96.7 99.3	0.77	181.1 > 153.1	10	292.1 > 181.1 181.1 > 56.0	8 24
Famoxadone	F	22.52	73.0 76.0	0.49	224.1 > 196.1	10	330.1 > 224.1 330.1 > 196.1	10 22
Fenamidone	F	18.00	69.2	0.40	238.1 > 103.1	22	268.1 > 180.1	16

			137.6				238.1 > 210.1	10
Fenarimol	F	19.00	83.1	0.84	251.0 > 139.0	14	330.0 > 139.0	8
			69.9				251.0 > 111.0	26
Fenbuconazole	F	20.12	139.9	0.44	198.1 > 129.1	10	129.1 > 102.1	18
			124.9				129.1 > 78.0	20
Fenclofos	I	12.84	92.2	0.16	284.9 > 269.9	16	286.9 > 271.9	18
			126.5				284.9 > 239.9	26
Fenoxanil	F	15.83	131.2	0.90	189.0 > 125.0	12	189.0 > 154.0	12
			113.9				293.1 > 155.0	22
Fenoxaprop-ethyl	H	19.32	103.3	0.36	361.1 > 288.1	12	288.1 > 119.1	12
			128.6				288.1 > 91.0	20
Fenoxycarb	IGR	17.87	115.0	0.15	255.1 > 186.1	10	186.1 > 109.1	14
			69.0				186.1 > 129.1	14
Fenpropathrin	I	17.98	79.3	0.81	181.1 > 152.1	22	265.1 > 210.1	12
			96.4				181.1 > 127.1	28
Fenpropimorph	F	13.59	106.0	0.66	128.1 > 70.0	10	128.1 > 110.1	8
			134.7				128.1 > 84.0	18
Fensulfothion	I	16.04	93.8	0.13	293.0 > 125.0	14	293.0 > 153.0	8
			87.1				293.0 > 141.0	12
Fenthion	I	13.51	139.4	0.37	278.0 > 109.0	20	278.0 > 169.0	14
			137.8				278.0 > 125.0	20
Fentitrothion	I	13.11	114.5	0.66	277.0 > 260.0	6	277.0 > 109.1	14
			107.3				260.0 > 125.1	12
Fenvalerate (two isomers)	I	21.35 / 21.55	116.4	0.69	225.1 > 119.1	20	225.1 > 147.1	10
			113.0				419.1 > 225.1	6
Fenvalerate-d7	Internal STD	21.52	103.6	-	426.2 > 225.1	6	426.2 > 174.1	14
			124.9					
Fluacrypyrim	I	16.32	118.0	0.63	320.1 > 183.1	16	352.1 > 188.1	10
			118.0				352.1 > 215.1	12

Flucythrinate (two isomers)	I	20.62 / 20.82	115.4 114.8	0.80	199.1 > 157.1	10	157.1 > 107.1 199.1 > 107.1	12 22
Flufenpyr-ethyl	H	15.98	83.0 123.2	0.16	408.0 > 345.0	16	408.0 > 321.0 408.0 > 305.0	14 18
Flumiclorac-pentyl	H	22.28	110.2 101.6	0.33	423.1 > 318.1	12	308.1 > 280.1 423.1 > 308.1	10 14
Flumioxazin	H	21.32	138.1 129.8	0.50	287.1 > 259.1	12	354.1 > 326.1 354.1 > 176.1	8 20
Fluquinconazole	F	19.74	70.5 72.8	0.22	340.0 > 298.0	20	340.0 > 313.0 342.0 > 300.0	14 22
Fluridone	H	20.99	113.3 71.3	0.66	328.1 > 259.0	24	328.1 > 313.0 328.1 > 127.0	22 24
Flusilazole	F	15.51	139.8 131.6	0.66	233.1 > 165.1	14	206.1 > 151.1 233.1 > 152.1	16 14
Fluthiacet-methyl	H	23.65	92.8 131.9	0.43	403.0 > 56.0	24	403.0 > 84.0 405.0 > 56.0	14 24
Fluvalinate	I	21.47 / 21.53	76.4 102.6	0.20	250.1 > 55.0	20	250.1 > 200.0 252.1 > 55.0	20 20
Fonofos	I	11.49	139.8 96.5	0.78	137.1 > 109.1	8	246.0 > 137.1 246.0 > 109.1	6 18
Formothion	I	12.16	110.1 92.3	0.25	170.0 > 93.0	8	224.0 > 125.0 170.0 > 63.0	18 22
Furametpyr	F	18.23	95.3 138.7	0.15	157.1 > 76.0	20	298.1 > 176.1 298.1 > 123.1	24 22
Halfenprox	I	20.53	100.8 126.5	0.52	263.0 > 117.0	14	263.0 > 235.0 263.0 > 129.0	14 26
Hexazinone	H	16.98	71.1 71.8	0.66	171.1 > 71.0	16	171.1 > 85.0 128.1 > 83.0	16 10
Imibenconazole	F	23.20	110.6	0.59	253.0 > 82.0	10	253.0 > 55.0	24

			74.7				375.0 > 260.0	22
Indanofan	H	18.09	85.7	0.36	139.0 > 111.0	14	139.0 > 75.0	26
			84.3				310.1 > 139.0	6
Indoxacarb	I	21.96	135.6	0.44	218.0 > 203.0	10	264.0 > 176.0	14
			70.3				218.0 > 134.0	22
Iprobenfos	I	12.06	98.4	0.88	204.0 > 91.0	8	204.0 > 122.0	12
			136.1				204.0 > 171.0	6
Iprobenfos-d7 (ISTD)	Internal STD	12.01	134.3	-	211.1 > 97.1	8	211.1 > 127.1	18
Iprodione	F	17.63	126.4	0.49	314.0 > 245.0	12	314.0 > 56.0	22
			73.5				316.0 > 247.0	12
Isazophos	I	11.77	73.9	0.82	257.0 > 162.0	8	257.0 > 119.0	18
			73.5				285.0 > 161.0	12
Isocarbophos	I	13.65	136.4	0.61	289.1 > 136.0	14	230.0 > 212.0	10
			71.3				230.0 > 198.0	10
Isofenphos oxon	I	13.61	86.6	0.82	229.1 > 201.0	10	201.0 > 121.0	20
			68.3				229.1 > 121.0	26
Isoprocarb	I	8.81	73.0	0.18	136.0 > 121.0	10	121.0 > 77.0	22
			112.1				121.0 > 103.0	12
Isoxadifen-ethyl	H	16.61	133.5	0.88	294.1 > 204.0	16	204.0 > 177.0	18
			80.7				204.0 > 151.0	28
Isoxathion	I	15.75	77.8	0.97	177.1 > 130.1	10	177.1 > 116.1	12
			74.2				313.1 > 177.1	8
Isoxathion-d10 (ISTD)	Internal STD	15.67	134.6	-	323.1 > 178.0	8	323.1 > 105.0	18
Kresoxim-methyl	F	15.54	120.1	0.74	206.1 > 131.1	14	206.1 > 116.1	6
			80.4				131.1 > 89.0	28
Lenacil	H	16.82	71.5	0.67	153.1 > 136.1	14	153.1 > 82.1	16
			123.2				153.1 > 110.1	16

Leptophos	F	18.48	124.0 109.9	0.22	376.9 > 361.9	24	374.9 > 359.9 376.9 > 268.9	24 36
Malathion	I	13.29	118.8 94.6	0.80	173.1 > 99.0	14	173.1 > 127.0 158.1 > 125.0	6 10
MCPA-thioethyl	H	11.93	79.3 113.6	0.32	155.0 > 125.0	10	244.0 > 75.0 155.0 > 89.0	14 22
MCPB-ehtyl	H	12.21	84.6 109.4	0.78	115.1 > 87.0	8	115.1 > 45.0 211.1 > 155.1	18 10
Mefenacet	H	18.68	93.4 131.6	0.74	192.0 > 136.0	14	192.0 > 109.0 148.0 > 120.0	24 8
Mefenpyr-diethyl	H	17.44	89.4 101.8	0.38	253.0 > 189.0	28	299.0 > 253.0 253.0 > 163.0	10 24
Mepronil	F	16.44	91.4 83.9	0.21	119.1 > 91.0	12	119.1 > 65.0 269.1 > 119.1	24 14
Methacrifos	I	8.37	82.3 76.2	0.61	208.0 > 180.0	8	240.0 > 208.0 208.0 > 110.0	4 18
Methoxychlor	I	17.97	131.0 69.0	0.77	227.1 > 169.1	24	227.1 > 212.1 227.1 > 141.1	14 28
Metolachlor	H	13.39	70.0 129.2	0.52	162.1 > 133.1	16	238.1 > 162.1 238.1 > 133.1	12 26
Metolachlor-d6 (ISTD)	Internal STD	13.36	83.7 130.5	-	242.2 > 166.1	16	242.2 > 134.1	26
Metominostrobin	F	15.58	103.7 69.0	0.22	191.0 > 160.0	10	238.1 > 210.1 191.0 > 133.0	14 20
Metribuzin	H	12.48	115.2 125.8	0.23	198.1 > 82.0	14	198.1 > 110.1 144.1 > 128.1	10 8
Mevinphos	I	7.58	116.5 111.8	0.59	127.0 > 109.0	12	192.0 > 127.0 127.0 > 95.0	12 18
Molinate	H	8.91	92.5	0.36	126.1 > 55.0	14	187.1 > 126.1	6

			98.7				126.1 > 83.0	6
Napropamide	H	18.18	83.8	0.73	128.1 > 72.0	6	100.0 > 72.0	8
			124.3				128.1 > 100.0	8
Nereistoxin	I	6.28	134.8	0.25	149.1 > 71.1	8	149.1 > 102.1	6
			76.0				103.1 > 70.1	8
Nitralin	H	17.31	107.8	0.69	316.1 > 274.0	8	274.0 > 169.0	12
			103.1				274.0 > 216.0	8
Nitrofen	H	15.86	86.5	0.93	202.0 > 139.0	24	282.9 > 253.0	12
			112.3				282.9 > 162.0	24
Norflurazon	H	16.71	93.8	0.74	145.0 > 95.0	18	303.0 > 145.0	22
			134.0				145.0 > 75.0	28
Omethoate	I	9.39	109.1	0.43	156.0 > 110.0	8	110.0 > 79.0	10
			120.9				156.0 > 79.0	22
Orthophenyl phenol	F	8.67	105.0	0.16	170.1 > 141.1	24	141.1 > 115.1	18
			116.1				170.1 > 115.1	28
Oxadixyl	F	16.15	77.3	0.11	163.1 > 132.1	8	132.1 > 117.1	18
			70.5				163.1 > 117.1	24
Oxpoconazole	F	19.81	74.5	0.94	294.1 > 179.1	12	294.1 > 197.10	6
			72.0				208.1 > 69.0	20
Oxyfluorfen	H	15.51	87.6	0.19	252.0 > 196.0	22	361.0 > 300.0	14
			119.1				361.0 > 317.0	6
Parathion	I	13.58	109.7	0.40	139.0 > 109.0	8	291.1 > 109.0	14
			73.0				291.1 > 137.0	6
Pentoxazone	H	18.43	100.5	0.86	285.0 > 70.0	14	287.0 > 70.0	18
			77.3				285.0 > 187.0	10
Permethrin	I	19.59 / 19.72	122.4	0.16	183.1 > 153.1	14	183.1 > 168.1	14
			68.9				183.1 > 165.1	14
Phenisobromolate	I	17.85	122.3	0.59	340.9 > 182.9	18	340.9 > 184.9	20
			137.6				340.9 > 157.0	30

Phorate	I	10.53	119.0 102.8	0.27	260.0 > 75.0	8	231.0 > 129.0 231.0 > 175.0	24 12
Phosalone	I	18.45	110.8 73.2	0.42	182.0 > 111.0	14	182.0 > 138.0 182.0 > 102.0	8 14
Phosmet	I	17.76	115.9 107.7	0.47	160.0 > 77.0	24	160.0 > 133.0 160.0 > 105.0	14 18
Phosphamidon	I	12.31	116.5 114.6	0.21	127.1 > 109.1	12	127.1 > 95.1 264.1 > 127.1	18 14
Picolinafen	H	17.85	124.1 98.3	0.11	376.1 > 239.1	12	238.1 > 145.1 376.1 > 266.1	24 12
Piperonyl butoxide	I	17.29	128.4 72.8	0.37	176.1 > 131.1	12	176.1 > 117.1 176.1 > 103.1	20 24
Pirimiphos-methyl	I	13.07	70.5 137.8	0.99	290.1 > 125.0	22	290.1 > 233.1 305.1 > 180.1	12 8
Prohydrojasmon	PGR	11.76	105.5 109.9	0.33	153.1 > 97.0	10	184.1 > 83.0 153.1 > 83.0	8 18
Prometryn	H	12.85	124.7 107.8	0.59	226.1 > 184.1	10	241.2 > 184.1 241.2 > 58.0	12 14
Propachlor	H	9.60	117.7 109.1	0.84	120.0 > 77.0	20	176.1 > 57.0 120.0 > 92.0	8 8
Propanil	H	12.42	86.7 94.0	0.81	217.0 > 161.0	10	160.9 > 99.0 160.9 > 90.0	24 22
Propargite	I	17.19	115.6 106.5	0.69	135.1 > 107.1	16	135.1 > 77.0 135.1 > 95.0	24 14
Propazine	H	11.19	78.2 77.2	0.41	125.0 > 89.0	18	125.0 > 99.0 229.1 > 58.0	18 14
Propyzamide	H	11.49	80.6 77.7	0.62	172.9 > 144.9	16	172.9 > 109.0 172.9 > 74.0	26 28
Pyraclofos	I	19.25	135.0	0.53	194.0 > 138.0	22	360.1 > 194.0	14

			79.9				360.1 > 139.0	14
Pyraclostrobin	F	21.45	125.1	0.50	132.1 > 77.0	20	164.1 > 132.1	14
			101.5				132.1 > 104.1	12
Pyraflufen-ethyl	H	16.86	76.7	0.39	412.0 > 349.0	16	412.0 > 289.0	24
			72.8				412.0 > 307.0	24
Pyrazophos	F/I	18.97	99.3	0.47	221.1 > 193.1	12	221.1 > 149.1	14
			111.5				221.1 > 177.1	16
Pyributicarb	H	17.51	133.1	0.90	165.1 > 108.1	10	165.1 > 93.0	24
			100.6				181.1 > 108.1	8
Pyridaben	I	19.75	78.9	1.00	147.1 > 117.1	22	147.1 > 132.1	14
			79.2				147.1 > 119.1	10
Pyridaphenthion	I	17.61	89.8	0.69	340.0 > 199.1	8	199.1 > 92.0	16
			138.8				199.1 > 77.0	24
Pyrimethanil	F	11.63	111.6	0.29	198.1 > 183.1	14	198.1 > 118.1	28
			69.5				198.1 > 158.1	18
Pyrimidifen	I	21.18	84.9	0.54	184.1 > 169.0	14	186.1 > 171.0	22
			129.2				184.1 > 86.0	24
Pyriminobac-methyl (two isomers)	H	15.99 / 16.81	108.2	0.14	302.1 > 256.1	18	302.1 > 230.1	18
			93.3				302.1 > 83.0	28
Pyriproxyfen	I	18.61	124.8	0.96	136.1 > 78.0	20	136.1 > 96.0	14
			71.7				226.1 > 186.1	14
Quinoclamine	H	13.33	89.8	0.20	207.0 > 172.0	12	172.0 > 89.0	24
			90.7				172.0 > 128.0	10
Quinoxifen	F	16.79	78.1	0.65	237.1 > 208.1	28	307.1 > 237.1	22
			90.1				307.1 > 272.1	8
Quizalofp-ethyl	H	20.64	138.9	0.34	372.1 > 299.1	14	299.1 > 255.1	18
			115.5				299.1 > 91.1	22
Resmethrin	I	17.35	68.2	0.69	143.1 > 128.1	10	171.1 > 143.1	6
			85.1				171.1 > 128.1	14

Silafluofen	I	20.92	80.2 110.4	0.47	286.1 > 258.1	14	286.1 > 207.1 258.1 > 215.1	14 22
Simazine	H	11.03	75.0 130.0	0.60	201.1 > 173.1	6	201.1 > 186.1 186.1 > 91.0	6 8
Simeconazole	F	12.63	87.5 122.7	0.53	211.1 > 195.1	4	195.1 > 75.0 211.1 > 121.1	18 14
Spirodiclofen	I	19.48	134.5 113.5	0.91	312.0 > 109.0	20	312.0 > 259.0 312.0 > 277.0	12 6
Spiroxamine	F	12.65	76.5 87.9	0.92	100.1 > 58.0	12	100.1 > 72.0 100.1 > 99.1	8 14
Sulfotep	I	10.29	68.4 120.5	0.14	322.0 > 202.0	10	322.0 > 174.0 322.0 > 294.0	18 4
Sulprofos	I	16.49	139.7 109.4	0.19	156.0 > 141.0	18	322.0 > 156.0 156.0 > 108.0	8 28
Swep	H	11.14	103.1 81.6	0.29	186.9 > 123.9	24	218.9 > 186.9 218.9 > 173.9	8 14
Tebuconazole	F	17.16	129.9 102.0	0.57	250.1 > 125.1	22	125.1 > 89.0 250.1 > 153.1	18 12
Tebuftenpyrad	I	18.08	138.4 132.8	0.29	333.1 > 171.1	20	333.1 > 276.1 318.1 > 131.1	8 18
Tebupirimphos	I	12.02	137.2 110.4	0.11	261.1 > 137.1	18	318.1 > 152.1 261.1 > 153.1	14 14
Tefluthrin	I	11.83	72.0 116.7	0.78	177.0 > 127.1	16	177.0 > 137.1 197.0 > 141.1	16 14
Terbacil	H	11.76	132.3 110.4	0.34	161.0 > 144.0	14	161.0 > 88.0 117.0 > 76.0	20 8
Terbucarb	I	12.43	77.5 98.9	0.80	220.2 > 205.2	12	205.2 > 57.1 205.2 > 177.2	18 8
Terbufos	I	11.40	121.7	0.49	231.0 > 128.9	26	231.0 > 174.9	14

			77.4				231.0 > 202.9	8
Terbutryn	H	13.11	126.2	0.19	241.2 > 185.1	6	241.2 > 170.1	14
			136.3				226.1 > 71.0	16
Tetraconazole	F	13.64	99.5	0.68	336.0 > 204.0	28	336.0 > 218.0	14
			82.8				336.0 > 164.0	24
Tetradifon	I	18.34	110.2	0.37	226.9 > 199.0	16	355.9 > 159.0	18
			112.9				355.9 > 228.9	12
Tetramethrin (two isomeers)	I	17.69 / 17.84	126.4	0.29	164.1 > 107.1	14	164.1 > 77.0	22
			100.2				164.1 > 135.1	8
Thenylchlor	H	17.08	108.1	0.59	288.1 > 141.1	14	141.1 > 126.1	16
			119.7				141.1 > 97.0	24
Thiobencarb	H	13.45	131.9	0.37	257.1 > 100.0	8	125.0 > 89.0	18
			89.6				257.1 > 72.0	20
Thiometon	I	10.77	129.8	0.10	88.0 > 60.0	8	125.0 > 47.0	14
			68.6				125.0 > 79.0	10
Tolfenpyrad	I	22.63	80.4	0.63	383.1 > 171.1	20	211.1 > 91.0	24
			122.6				383.1 > 145.1	12
Triadimefon	H	13.64	131.6	0.16	208.1 > 181.0	10	208.1 > 111.0	22
			75.8				208.1 > 127.0	14
Triallate	H	11.92	94.5	0.19	268.1 > 184.0	20	270.1 > 186.0	20
			121.5				268.1 > 226.0	14
Triazophos	I	16.46	124.4	0.13	161.0 > 134.0	8	161.0 > 106.0	14
			133.6				257.0 > 162.0	8
Trichlamide	F	20.19	88.7	0.88	148.1 > 121.1	14	121.1 > 65.0	18
			104.3				148.1 > 93.0	22
Triclofos-methyl	F	12.64	118.7	0.79	264.9 > 249.9	14	264.9 > 93.0	24
			109.6				264.9 > 219.9	22
Trifloxystrobin	F	16.71	99.2	0.81	222.1 > 190.1	4	222.1 > 130.1	12
			118.6				222.1 > 162.1	10

Zoxamide	F	17.44	104.8	0.64	187.0 > 159.0	16	187.0 > 123.0	24
			70.2				258.1 > 187.0	14

I: insecticide, H: herbicide, F: fungicide, IGR: insect growth regulator, PGR: plant growth regulator

CHAPTER 3

Characteristics of cytochrome P450-dependent
metabolism against acetamiprid in the musk shrew
(*Suncus murinus*)

Abstract

Soricidae spp. (shrews) play an essential role in soil ecosystems and, due to their habitat and behavior, are exposed to soil pollutants, such as pesticides. Still, toxicity risk in *Soricidae* spp. has not been appropriately assessed. In this study, the musk shrew (*Suncus murinus*) was used as a model organism for toxicity assessment in *Soricidae*. Considering their carnivorous diet, it is reasonable to assume that the musk shrew has unique metabolic traits that are different from those of other common experimental models. This study describes the cytochrome P450 (CYP)-dependent metabolism affected by acetamiprid (ACP), a neonicotinoid insecticide. Pharmacokinetics analysis, an *in vitro* metabolic assay, and genetic analysis of CYP were performed and compared with data from mice and rats. Through phylogenetic and syntenic analyses, three families of CYP were identified in the musk shrew. Pharmacokinetic analysis showed that the blood concentration of ACP decreased more quickly in musk shrews than in mice. Moreover, the *in vitro* metabolic assay suggested more efficient metabolic responses toward ACP in musk shrews than in mice or rats. One of the CYP2A isoforms in musk shrews might be linked to a better ACP metabolism. From the results above, I describe novel metabolic traits of the musk shrew. Future research on recombinant CYP enzymes is necessary to fully understand CYP-dependent metabolism of xenobiotics in musk shrews.

Keywords

metabolizing enzyme, neonicotinoid, pesticide, *Soricidae*, wildlife

Highlights

- Phylogenetic and syntenic analysis revealed unique characteristics of cytochrome P450 (CYP) genes in musk shrews compared to other mammalian species.
- Musk shrews exposed orally to acetamiprid (ACP) showed a rapid decrease in blood concentrations of ACP compared to mice.
- Musk shrews showed a more efficient metabolic response to ACP compared to mice and rats, as indicated by higher V_{max}/K_m ratios in the *in vitro* metabolic assay.
- A significant involvement of specific CYP isoforms, particularly CYP2A, in the metabolism of ACP was indicated in musk shrews.

1. Introduction

The family *Soricidae* includes more than 400 species of shrews (Schmidly et al., 2021). Most *Soricidae* spp. feed mainly on earthworms, insects, and reptiles and play essential roles as top predators in soil ecosystems (Schmidly et al., 2021). *Suncus murinus* (musk shrews) of the *Soricidae* family are highly carnivorous. Their diet includes insects, arthropods, and earthworms (Browett et al., 2021). In recent years, the population decrease of many *Soricidae* species has been reported worldwide (Kennerley et al., 2021), and one possible reason for this decrease is the direct exposure to pesticides in their soil habitat. For example, organochlorine insecticides were detected in wild shrews (Mathias et al., 2007). In addition, previous research indicates that xenobiotics, such as insecticides, can reach shrews through biomagnification within the food chain of the soil ecosystem; thus, high amounts of insecticides might accumulate in shrews as they are among the top predators in the soil ecosystem (Dell’Omo et al., 1999; Petkovsek et al., 2015). However, current risk assessments of insecticides target mainly human and experimental animals such as mice, rats, and rabbits as subjects (Khlebtsov et al., 2011; Rim, 2020; Proença et al., 2021); and there is a lack of research on insecticide-induced toxicity in *Soricidae* spp. Due to differences in the sensitivity toward xenobiotics among species (Hutchinson et al., 2014), toxicity assays using exclusive experimental rodents are inadequate for estimating toxicity in *Soricidae* spp. as the two groups are phylogenetically distinct (Holmberg, 2022), raising the importance of a risk assessment specific to the *Soricidae* family.

It has been reported that carnivorous mammals have lower metabolic rates than herbivorous mammals in metabolizing xenobiotic compounds. This is explained by herbivores’ adaptation to metabolizing plant-derived toxins (Court, 2013; Kakehi et al.,

2015). These metabolic differences suggest an incompatibility between musk shrews and common experimental animals. Although experiments on shrews can be challenging due to the shrews' high sensitivity to changes in basal metabolic rate (Genoud, 1985; Taylor, 1988), musk shrews can be kept and bred in a manner similar to rats. Several strains have already been established to date (Ishikawa et al., 1987; Oda et al., 1992; Uchino et al., 2001), thereby guaranteeing the procurement of enough shrews to achieve the sample size necessary for obtaining reliable experimental results. Since the whole genome information of the musk shrew is available (Database of *Suncus murinus* Genome Project, authentication is needed for use: <https://suncus.nig.ac.jp/suncus/>), a genetic comparison between all these species can be performed. With these characteristics, musk shrews can be considered a valuable model for evaluating the toxic effects of environmental pollutants on wild shrews. Metabolic capacities are essential factors in determining a species' sensitivity toward xenobiotics. Most xenobiotics are metabolized by the cytochrome P450 (CYP), a xenobiotic metabolizing enzyme in mammalian livers (Wang et al., 2019). Cytochromes from CYP1-3 families, more specifically, are vital in the detoxification of xenobiotics (Rendic et al., 2015), and thus, investigations on CYP metabolic capabilities have been performed in humans and experimental animals to estimate the sensitivities toward xenobiotics (Bogacz et al., 2012; Esteves et al., 2021). Previous research has shown that CYP-dependent metabolic ability of musk shrew might be slightly lower than that of mice or rats (Mizokami et al., 1988; Mushiroda et al., 2000). However, details of each CYP isoform, such as genetic characterization, have not been reported yet. A detailed description of the CYP isoforms in musk shrews will help establish a novel risk assessment method for this species.

Among many environmental pollutants, insecticides can be the most influential xenobiotics on wild mammals (Guitart et al., 2010; Lakhani et al., 2015; Bertero et al., 2020), and acetamiprid (ACP) is one of the most abundantly used insecticides for agriculture in many countries (Simon et al., 2015). To elucidate the characteristics of CYP-dependent metabolism in musk shrews, I focused on ACP, a neonicotinoid insecticide (NNI) that can be metabolized by CYPs for detoxification (Casida, 2011; Marfo et al., 2015; Khidkhan et al., 2021). NNIs, including ACP, have lower toxicity in mammals and selectively act on insects by targeting their nicotinic acetylcholine receptors (Tomizawa et al., 2003; Wang et al., 2018). Nonetheless, unknown NNI-induced toxicity in mammals have been getting more interest in environmental toxicology (Roy et al., 2020; Kaczyński et al., 2021). The NNIs imidacloprid and thiamethoxam are metabolized by CYP3A4, 2C19, 2B6, and 2A6 in humans (Shi et al., 2009), but there is currently no information about which CYP isoforms are involved in the metabolism of ACP. This study aims to describe the characteristics of CYP-dependent xenobiotic metabolism in the musk shrew in the presence of ACP through pharmacokinetics, metabolic, and genetic analysis of *CYP* genes.

2. Materials and Methods

2.1. Phylogenetic analysis of CYP genes

Phylogenetic analysis was performed using *CYP1*, *CYP2*, and *CYP3* genes. *CYP* genes of the common shrew (*Sorex araneus*), mouse, rat, and human were accessed through the National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>). *CYP* genes of *Suncus murinus* were retrieved from the Genome Project database (<https://suncus.nig.ac.jp/suncus/>) using the basic local

alignment search tool (BLAST). Human *CYP1A1*, *1B1*, *2A6*, *2B6*, *2C8*, *2D6*, *2E1*, *2F1*, *2J2*, *2R1*, *2S1*, *2U1*, *2W1*, and *3A4* were used as query sequences for BLASTN (E-value = 10^{-10}). The *CYP* genes used in this phylogenetic analysis are shown in Table S3-1, S3-2, and S3-3. The amino acid sequences were aligned using MUltiple Sequence Comparison by Log-Expectation (MUSCLE); model selection and construction of maximum likelihood trees (bootstrapping = 500) was performed using Molecular Evolutionary Genetics Analysis X (MEGAX) (Kumar et al., 2018). The best model (Jones, Taylor, and Thorton + G model) was used to plot the phylogenetic tree. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). As outgroup, the following genes were used: human *CYP2A6*, *2B6*, *2C19*, *3A4*, and *3A5* for CYP1 family phylogenetic tree; HUMAN *1A1*, *1A2*, and *1B1* for CYP2 family phylogenetic tree; human *CYP1A1*, *1A2*, *1B1*, *2BA6*, *2B6*, and *2C19* for CYP3A family phylogenetic tree. This analysis involved 242, 452, and 285 amino acid sequences in CYP1, 2, 3 phylogenetic trees, respectively. There was a total of 143, 246, and 168 positions in the final dataset for the CYP1, 2, and 3 families in the phylogenetic tree, respectively.

2.2. Syntenic analysis of *CYP* genes

Syntenic analysis of *CYP* genes for mammalian species used in the phylogenetic analysis was performed using Genome Data Viewer of NCBI (<https://www.ncbi.nlm.nih.gov/genome/gdv/>) and JBROWSE of *Suncus murinus* Genome Project database (<https://suncus.nig.ac.jp/suncus/>). The following latest genome assemblies were applied: musk shrew (*Suncus murinus*) Assembly ID r131217,

common shrew (*Sorex araneus*) Assembly ID 418318, Annotation Release 101, mouse (*Mus musculus*) Assembly ID 7358741, Annotation Release 109, rat (*Rattus norvegicus*) Assembly ID 9079111, Annotation Release 108, and human (*Homo sapiens*) Assembly ID 11968211, Annotation Release 110. The annotation of the musk shrew *CYP* genes was conducted by mapping the RNA-sequencing (RNA-seq) data of different organs, including the liver, kidney, and small intestine. A total of 15 protein sequences were used as query sequences (CYP1, 16 CYP2A, 25 CYP2B, 52 CYP2C, 19 CYP2D, 4 CYP2E, 5 CYP2F, 4 CYP2G, 21 CYP2J, 4 CYP2R, 5 CYP2S, 3 CYP2T, 4 CYP2U, 4 CYP2W, and 22 CYP3A).

2.3. *CYP* expression levels by RNA-seq

Raw RNA-seq data from a female adult musk shrew liver (n = 1) was provided by the National Institute of Genetics (NIG, Shizuoka, Japan). Library preparation for sequencing was performed using TruSeq Strand mRNA Sample Prep Kit (Illumina, CA, USA). The libraries were sequenced using HiSeq 2500 (Illumina) as paired-end 100-bp. In total, 7,581,572 paired sequences were used.

QIAGEN CLC Main Workbench 21.0 (QIAGEN, Aarhus, Denmark) was employed for the following analysis. Trim Reads 2.6 was used for adapter sequences trimming (quality limit = 0.01, ambiguous nucleotides trimming, automatically adapter trimming). The genome assembly sequences of the musk shrew provided by the NIG (ID: r131217) were used as a reference for mapping (N50: 3.63 Mb; number of contigs: 143,917; total length: 2.37 Gb; average length: 181 kb; maximum length: 13.3 Mb; minimum length: 1,000 bp). RNA-Seq Analysis (version 2.6, QIAGEN) was used for mapping. Annotation files provided by NIG (24,589 genes and 63,135 transcripts

information) were used for masked gene mapping. The resulting rate of reads mapped in pairs was 81.3%. RNA-Seq Analysis 2.6 was also used to calculate the transcripts per million (TPM) for each CYP. Detailed parameters of trimming and mapping are shown in Table S3-4.

2.4. Animals and treatments

Musk shrews (Jic:SUN-Her strain, 7-weeks-old) were purchased from Tokyo Laboratory Animal Science (Tokyo, Japan). Six-week-old C57BL/6JJcl mice were obtained from CLEA Japan (Tokyo, Japan). All animals used in the present study were male. Animals were kept under a 12/12-hour light/dark cycle. Musk shrews were housed separately. For musk shrews, the temperature was set between a 24°C and 26°C range, and for mice, between 20°C and 23°C. Musk shrews were fed food for trout 5P (FEED ONE, Kanagawa, Japan) and mice were fed CE-2 (CLEA Japan). Water and food were provided to the animals *ad libitum*. The animals were not fasted prior to ACP administration.

ACP (20 mg·kg⁻¹; Cosmo Bio Co., Ltd., Tokyo, Japan) was orally administered to musk shrews (n = 7, body weight [mean ± SD]: 63.9 ± 2.9 g) and mice (n = 7, body weight: 21.7 ± 0.8 g) using a gavage. For mice, ACP was administered under anesthesia with isoflurane. No anesthesia was administered to musk shrews due to a regurgitating reflex caused by isoflurane. Blood (10 µL) was sampled from the tail vein through an incision and collected in a hematocrit tube at 5, 15, 30, 60, 120, 180, 240, 360, 720, and 1440 min after ACP exposure. A total of seven samples (n = 7) were collected from both musk shrews and mice.

The animal care and experiments procedures were performed according to the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Approval number: 21-0046, 21-0076). The animal experiments at the Graduate School of Hokkaido University were performed as per the Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

2.5. Extraction of ACP and its metabolites in blood

Blood (10 μ L) was added to 250 μ L of 0.1% formic acid in acetonitrile (ACN), 10 μ L of 100 parts per billion (ppb) ACP-d₃ (Sigma-Aldrich, Darmstadt, Germany), and 10 μ L of 100 ppb des methyl (dm)-ACP-d₃ (Sigma-Aldrich). Mixtures were centrifuged at $10,000 \times g$ for 10 min. Then, 100 μ L of supernatant was mixed with 300 μ L of 0.1 % formic acid in distilled water (DW) and transferred to high-performance liquid chromatography vial. Recovery rate and matrix effect of ACP and its metabolites were determined using blank blood samples as shown in Table S3-5.

2.6. Preparation of liver microsome fractions

Livers of musk shrews, mice, and rats were removed immediately after euthanasia and frozen in liquid nitrogen. The samples were stored at -80°C until the subsequent assay. Detailed information on the samples used in this study is shown in Table S3-6. Liver microsomal fractions of musk shrews, mice, and rats (n = 4) were prepared using a protocol by Omura et al. (1964) with slight modifications. The livers were homogenized in about three times the volume of 0.1 M potassium phosphate buffer (KPB, pH 7.4) and then centrifuged at $9,000 \times g$ for 20 min at 4°C. The

supernatant was filtered through sterile gauze and centrifuged twice at $34,000 \times g$ for 60 min at 4°C. Microsomal pellets at the bottom of the centrifuge tubes were resuspended in 0.1 M KPB and stored at -80°C until used. Pooled human microsome fractions were purchased from BioIVT (Westbury, NY, USA). The microsomal protein concentrations were determined by the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Waltham, MA, USA). The CYP concentrations were measured using the method reported by Omura et al. (1964).

2.7. *In vitro* metabolic assay of ACP

The CYP-dependent metabolic assay was conducted using liver microsomes of musk shrews, mice, and rats following the methodology of Shinya et al. (2022). The incubation mixture consisted of liver microsomes ($3 \text{ mg} \cdot \text{mL}^{-1}$ of protein concentrations), glucose-6-phosphate (5 mM), MgCl_2 (3 mM in final concentration), and KPB (0.1 M, pH 7.4). As a substrate, ACP was added at two levels of concentration to KPB: low concentrations (1.25, 2.5, 5, 10, 20, and 40 μM) and high concentrations (12.5, 25, 50, 100, 200, 400, 600, 800, and 1000 μM). Those different levels were used to simulate an *in vivo* exposure and calculate kinetic parameters. For the enzymatic reaction, samples were incubated at 37°C for 5 min. Then, glucose-6-phosphate dehydrogenase ($2 \text{ IU} \cdot \text{mL}^{-1}$) and nicotinamide adenine dinucleotide phosphate hydrogen (NADPH; 0.5 mM) were added to each sample and returned to incubation for 30 min. After the incubation period, 200 μM of 1% formic acid in ACN was mixed to terminate the enzymatic reaction. Samples were then centrifuged at $15,000 \times g$ for 10 min. The supernatant was filtered through a GL Chromato Disk sample filter (pore size 0.2 μM ; GL Sciences, Tokyo, Japan). All assays were conducted in duplicate for each concentration. A negative

control (without substrate) was used for each sample. The velocity was represented as $\text{pmol}^{-1} \cdot \text{min}^{-1} \cdot \text{nmol CYP}$.

2.8. *In vitro* CYP inhibition assay

It is known that NNIs imidacloprid and thiamethoxam are metabolized by CYP3A4, 2C19, 2B6, and 2A6 in humans (Shi et al., 2009), but there is no information about which CYP isoforms are involved in the metabolism of the ACP. To reveal which CYP isoforms catalyze ACP metabolism in humans, an *in vitro* inhibition assay was performed using specific CYP inhibitors, including ketoconazole for CYP3A4 (Dilmaghanian et al., 2004), ticlopidine for CYP2C19 (Ha-Duong et al., 2001), 2-phenyl-1-2-(1-piperidinyl) propane for CYP2B6 (Walsky et al., 2007), and tranlycypromine hydrochloride for CYP2A6 (Zhang et al., 2001). All inhibitory compounds were purchased from FUJIFILM Wako Chemicals (Tokyo, Japan). The incubation mixture contained liver microsomes ($1 \text{ mg} \cdot \text{mL}^{-1}$ of protein concentrations), ACP ($10 \text{ } \mu\text{M}$), inhibitor, glucose-6-phosphate (5 mM), MgCl_2 (3 mM in final concentration), and KPB (0.1 M , pH 7.4). For the inhibitors, the following nine concentrations were applied: 0.001 , 0.01 , 0.1 , 0.5 , 1 , 5 , 10 , 50 , and $100 \text{ } \mu\text{M}$ in final concentration. While ticlopidine and tranlycypromine hydrochloride were dissolved in 0.1 M KPB, ketoconazole and 2-phenyl-1-2-(1-piperidinyl) propane were dissolved in 1.6% methanol. As previously reported by Iwase et al. (2006), methanol concentrations up to 3% do not influence the inhibitory effect of ketoconazole in the *in vitro* assays. An incubation mixture without any inhibitors was also prepared to serve as the control. Incubation was performed by the same method as that of the *in vitro* metabolic assay described in section 2.7. The metabolites were measured using a liquid

chromatography-mass spectrometer (LC/MS/MS) as described in the next section. The amounts of produced dm-ACP compared to the control were represented in percentages.

2.9. Measuring ACP and its metabolites by LC/MS/MS

An LC/MS/MS (6495 triple quad LC/MS; Agilent Technologies, Santa Clara, CA, USA) equipped with SM-C18 column (3.0×100 mm, $\phi 3$ μ m, Imtakt, Kyoto, Japan) was used to measure ACP and its metabolites. Des methyl-ACP (dm-ACP; Sigma Aldrich), des cyano-ACP (dc-ACP), des methyl-des cyano-ACP (dm-dc-ACP), N-acetyl-ACP (N-ace-ACP), and N-acetyl-des methyl-ACP (N-ace-dm-ACP) were determined as metabolites of ACP (Khidkhan et al., 2021). Dc-ACP, dm-dc-ACP, N-ace-ACP, and N-ace-dm-ACP were synthesized at Toho University. Mobile phase A was double DW. Mobile phase B comprised 0.1% of formic acid and 100 mM of ammonium acetate in 90% methanol (v/v). The applied gradient was as follows: $t = 0$ –1 min, 2% solvent B; $t = 8$ min, 80% solvent B; $t = 8$ –9.5 min, 98% solvent B. Injection volume, flow rate, and column temperature were 5 μ L, 0.35 mL $\cdot$ min $^{-1}$, and 60°C, respectively. Parameters for each compound are shown in Table S3-7. Signal to noise ratio (S/N) higher than nine was considered a detected peak. The analysis was performed using the internal standard method for the *in vivo* pharmacokinetics experiment, and the absolute calibration curve method was applied for the *in vitro* metabolic assay.

2.10. Data analysis

Non-compartmental pharmacokinetic analysis of the blood concentration was conducted using PKPlus 2.5 (Simulations Plus, CA, USA). The pharmacokinetic

parameters, including maximum concentration ($C_{\max}$), half-life ($T_{1/2}$), area under the curve (AUC), and clearance (CL), were estimated. For the analysis of *in vitro* metabolic assay, 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 version 8.0 for Windows (GraphPad Software, San Diego, CA, USA). Statistical analyses were conducted using the JMP® Pro 16 software (SAS Institute, Cary, NC, USA). Steel-Dwass test was performed to compare $V_{\max}/K_m$ ratio and CYP concentration among animal species. Student t-test was applied to compare pharmacokinetic parameters between musk shrews and mice. A *p*-value less than 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Phylogenetic and syntenic analysis of CYP genes

Phylogenetic tree and synteny using the musk shrew, common shrew, mouse, rat, and human for *CYP1*, *CYP2*, and *CYP3* genes are shown in Figure 3-1, 3-2, and 3-3, respectively. Based on the results of phylogenetic tree and synteny, the following *CYP* genes were identified in the musk shrew: two *CYP1A* (Transcript ID in *Suncus murinus* Genome Project database: R54996, R54997), one *CYP1B* (R56407), two *CYP2A* (R45726, R45729), six *CYP2B* (R26308, R26314, R26318, R35282, R45730, R45735), three *CYP2C* (R10892, R10897, R10901), two *CYP2D* (R44892, R64413), one *CYP2E* (R68058), one *CYP2F* (R45724), two *CYP2J* (R18107, R18081), one *CYP2R* (R10074), one *CYP2S* (R16164), and four *CYP3A* (R09733, R14459, R14460, R14466). The numbers of confirmed *CYP* genes in each species are shown in Table 3-1.

An orthologous relationship was confirmed between *CYP1* genes on musk shrew and other species (Figure 3-1). On the other hand, most *CYP2* genes, including *CYP2A*, *CYP2B*, *CYP2D*, and *CYP2J* from musk shrews and common shrews, formed isolated clusters from humans, rats, and mice (Figure 3-2). Moreover, as shown in Figure 3-3, two *CYP3* genes in musk shrews (R14460, R14466) were located in a different cluster from rats, mice, and humans. Two *CYP3* genes (R09733, R14459) showed an orthologous relationship with rat *Cyp3a73*.

3.2. mRNA expression levels of *CYP3A* genes

TPM of *CYP* genes in the liver of musk shrews' liver is shown in Figure 3-4. High expression levels (>50 in TPM) were observed in some *CYP* genes. The genes with the highest TPM are ranked as follows: R45729 (*CYP2A*, TPM = 3870), R10892 (*CYP2C*, TPM = 2309), R45735 (*CYP2B*, TPM = 1164), R68058 (*CYP2E*, TPM = 1041), R54997 (*CYP1A*, TPM = 796), R10897 (*CYP2C*, TPM = 417), R54996 (*CYP1A*, TPM = 301), R44892 (*CYP2D*, TPM = 298), R35282 (*CYP2B*, TPM = 231), R10901 (*CYP2C*, TPM = 54), and R64413 (*CYP2D*, TPM = 51). TPM values of other *CYP* genes were less than 10. No *CYP3A* genes showed significantly high expression levels.

3.3. Pharmacokinetics of the ACP on musk shrews and mice

The results of the pharmacokinetic analysis are shown in Figure 3-5 and Table 3-2. ACP, dm-ACP, N-ace-ACP, and N-ac-dm-ACP were detected among the metabolites. N-ace-ACP and N-ace-dm-ACP were detected exclusively in mice and at very low concentration levels ($C_{\max} = 7947 \pm 251 \text{ ng}\cdot\text{mL}^{-1}$ [dm-ACP], $2.44 \pm 0.38 \text{ ng}\cdot\text{mL}^{-1}$ [dc-ACP], $6.34 \pm 1.53 \text{ ng}\cdot\text{mL}^{-1}$ [N-ace-ACP], and $10.92 \pm 1.47 \text{ ng}\cdot\text{mL}^{-1}$ [N-ace-

dm-ACP]). Musk shrews showed significantly higher maximum concentrations of ACP ($C_{\max} = 11940 \pm 793 \text{ ng}\cdot\text{mL}^{-1}$) than mice ($C_{\max} = 7947 \pm 251 \text{ ng}\cdot\text{mL}^{-1}$) ($p = 0.012$). A significantly lower half-life was observed in musk shrews ($T_{1/2} = 93 \pm 9 \text{ min}$) than in mice ($T_{1/2} = 153 \pm 12 \text{ min}$) ($p = 0.007$). In dm-ACP, musk shrews showed significantly higher AUC than mice (musk shrew: $5452 \pm 748 \text{ }\mu\text{g}\cdot\text{h}\cdot\text{mL}^{-1}$, mice: $4427 \pm 671 \text{ }\mu\text{g}\cdot\text{h}\cdot\text{mL}^{-1}$) ($p = 0.024$). A high blood concentration of dm-ACP indicated that this is the main metabolite produced by ACP both in musk shrews and mice.

3.4. Concentrations of CYP in liver microsomes

The concentrations of CYP in the liver microsomes were determined and shown in Table 3-3. Musk shrews showed significantly lower CYP concentration than mice and rats (musk shrews: $0.55 \pm 0.064 \text{ nmol}\cdot\text{mg}^{-1}$ protein, mice: $0.77 \pm 0.019 \text{ nmol}\cdot\text{mg}^{-1}$ protein, $p = 0.011$, rats: $0.99 \pm 0.14 \text{ nmol}\cdot\text{mg}^{-1}$ protein, $p = 0.0003$).

3.5. CYP-dependent metabolic abilities against ACP in liver

Based on the result of pharmacokinetic analysis, I focused on dm-ACP as the most abundant metabolite. As shown in Figure 3-6a and Table 3-4, musk shrews showed significantly higher $V_{\max}/K_m$ ratio than mice and rats (musk shrews: $12.1 \pm 2.3 \text{ }\mu\text{L}\cdot\text{min}^{-1}\cdot\text{nmol CYP}^{-1}$, mice: $1.9 \pm 0.37 \text{ }\mu\text{L}\cdot\text{min}^{-1}\cdot\text{nmol CYP}^{-1}$, $p = 0.0014$, rat: $3.4 \pm 0.46 \text{ }\mu\text{L}\cdot\text{min}^{-1}\cdot\text{nmol CYP}^{-1}$, $p = 0.019$). The higher $V_{\max}/K_m$ ratio in musk shrews is due to their low K_m values (musk shrews: $61 \pm 9 \text{ }\mu\text{L}\cdot\text{min}^{-1}\cdot\text{nmol CYP}^{-1}$, mice: $125 \pm 19 \text{ }\mu\text{L}\cdot\text{min}^{-1}\cdot\text{nmol CYP}^{-1}$, rats: $388 \pm 76 \text{ }\mu\text{L}\cdot\text{min}^{-1}\cdot\text{nmol CYP}^{-1}$). At low ACP concentration, comparable to the blood concentration in the *in vivo* exposure test, musk shrews showed the highest velocity (Figure 3-6b).

3.6. Inhibition assay of CYP-dependent metabolism in human liver

The inhibition assay results using human liver microsome are shown in Table 3-5. Significant reduction of dm-ACP formation was observed for tranylcypromine, a CYP2A6 inhibitor. Ketoconazole, a CYP3A4 inhibitor, led to a slight reduction in dm-ACP formation at higher concentrations. No reduction was observed for other inhibitors.

4. Discussion

4.1. Genetic characteristics of CYPs in musk shrew

The orthologous relationship of CYP1 among species suggests that *CYP1* genes of *Soricidae* and *Muridae* (mice and rats) are derived from a common ancestral gene, which is consistent with previous studies where CYP1 is reported to be homologous among animal species. CYP1 can be considered a highly conserved isoform within the phylogenetic tree as CYP1 proteins share a strong structural similarity. These proteins also display similar specificities to their orthologous forms across different animal species (Ioannides et al., 2004). In contrast, *Soricidae* and *Muridae* CYP2 genes such as *CYP2A*, *2B*, *2D*, and *2J* were located separately in the phylogenetic tree, indicating that these genes have evolved independently. This result supports the findings of previous studies stating that the CYP2 family has evolved independently in different animal species, unlike the CYP1 family (Martignoni et al., 2006; Kitanovic et al., 2018a; Kitanovic et al., 2018b).

It is known that feeding habits affect the occurrence of *CYP* genes in mammalian species; carnivorous mammals have fewer *CYP* genes and thus a lower CYP-dependent metabolic ability (Kim et al., 2016). Also, as shown in this study, *CYP*

gene count supports the hypothesis of a link between feeding habits and the number of CYPs. Musk shrews and common shrews had a lower count of *CYP2D* and *CYP3A* genes than mice and rats (Table 3-1). Although the number of *CYP* genes does not always translate into metabolic capacities, musk shrews and common shrews might show lower activity in *CYP2D*- and *CYP3A*-dependent metabolic paths. In particular, low *CYP3A* abilities could be attributed to low mRNA expression levels of *CYP3A* in musk shrew. Although RNA-seq was conducted using a small number of samples ($n = 1$), low *CYP3A* expression has been confirmed not only in this study but also in a previous study (Mushiroda et al., 2000). It might be helpful to evaluate the metabolic abilities of *CYP3A* against typical substrates of human *CYP3A* proteins, such as erythromycin (Lewis et al., 2002), in shrews. Lower numbers of *CYP2C* and *2J* were confirmed in musk shrews. One explanation is the location of the gene sequence at an unscaffolded locus, causing no detection by either syntenic analysis or BLAST.

4.2. *CYP-dependent metabolism against ACP in musk shrews*

The highest $V_{\max}/K_m$ ratio was observed in musk shrews, indicating higher *CYP*-dependent metabolic activities against ACP in musk shrews compared to mice and rats. It is known that the toxicity of dm-ACP in mammals is lower than the ACP (Phogat et al., 2022). That suggests that musk shrews might be less sensitive to ACP than mice or rats, based on the higher metabolic abilities in their liver. The lower K_m value in musk shrews indicates a high substrate affinity to ACP (Alberty, 1952). Also, in comparison to the ACP exposure test *in vivo*, musk shrews showed the highest dm-ACP formation abilities. These results could be linked to the significantly shorter $T_{1/2}$ of musk shrews during *in vivo* pharmacokinetics analysis.

The *in vitro* CYP inhibition assay showed that CYP2A could be involved in ACP metabolism in humans. CYP2A isoforms metabolize the same chemicals among different animal species with low interspecies specificity. For example, CYP 2A catalyzes coumarin 7-hydroxylation in mice, rats, humans, dogs, and monkeys (Kobliakov et al., 1993; Bogaards et al., 2000; Donato et al., 2002). Therefore, it is possible that CYP2A in musk shrews also metabolizes ACP. One *CYP2A* gene (R45729) in this study showed the highest expression levels. Interestingly, the expression levels of CYP2A genes are generally lower than CYP2C and CYP3A, as seen in humans and experimental mammals (Martignoni et al., 2006). Although the sample size of RNA-seq analysis involving the musk shrews was small ($n = 1$), the high expression levels of CYP2A are consistent with the northern blotting results using musk shrew livers in the previous study (Mushiroda et al., 2000), and might lead to high metabolic abilities towards ACP in this species. In mammals, CYP2A is involved in xenobiotic metabolism, such as nicotine and coumarin (Martignoni et al., 2006). Musk shrews might be less sensitive to these compounds based on the possibility that they appear to have a high CYP2A metabolic capacity. To elucidate the details of CYP-dependent metabolic capacities in musk shrews, future testing should be performed using recombinant microsomes of each CYP protein.

4.3. Chemosensitivity against xenobiotics in musk shrews

Although high CYP-dependent metabolic ability against ACP, which was thought to be catalyzed by CYP2A, was confirmed in this study, previous studies have showed that CYP activity against some xenobiotics such as benzo[a]pyrene and 7-ethoxycoumarin is lower in musk shrews than in rats and mice (Mizokami et al 1988;

Miura et al., 1991). In humans, metabolism of benzo[a]pyrene and 7-ethoxycoumarin is mainly catalyzed by CYP1A1, CYP1A2, and CYP1B1 (Bukowska et al., 2022), and CYP1A2 and CYP2E1 (Shimada et al., 1999), respectively. Thus, these CYP isoforms of musk shrews might show lower metabolic abilities than those of other animal species. In this study, metabolic capacities related to only ACP were investigated; I did not evaluate the metabolism of other xenobiotics by other CYP isoforms. Differences in metabolic capacities in one or several CYP isoforms may alter the pharmacokinetics or sensitivities towards xenobiotics, which can increase chemical-induced risk. For example, sex differences in the expression level of CYP3A4 lead to differences in verapamil clearance between men and women (Fattore et al., 2009; Fillingim et al., 2009) and *Cyp2c* gene cluster knockout mice show higher blood concentrations of administered tolbutamide than normal mice (Scheer et al., 2012). It is necessary to evaluate the metabolic abilities of various CYP isoforms that catalyze environmental pollutants in musk shrews in the future.

5. Conclusions

In conclusion, this study elucidates the unique characteristics of CYP-dependent metabolism in musk shrews with respect to the neonicotinoid insecticide ACP. Through pharmacokinetic analysis, *in vitro* metabolic assays, and genetic analysis of CYP genes, it was found that musk shrews exhibit distinct metabolic responses to ACP compared to mice and rats. The musk shrews showed a more rapid decrease in ACP blood concentrations, a higher efficiency in ACP metabolism, and a significant involvement of specific CYP isoforms, particularly CYP2A, in ACP metabolism. These findings highlight the importance of considering species-specific metabolic traits in the toxicity

assessment of environmental contaminants and suggest that the musk shrew is a valuable model for assessing the toxic effects of pesticides in wildlife. Further research focusing on recombinant CYP enzymes is warranted to fully understand the CYP-dependent metabolism of xenobiotics in musk shrews and its implications for environmental toxicology.

Table 3-1: The numbers of confirmed CYP genes in each species

	Species				
	Musk shrew	Common shrew	Mouse	Rat	Human
CYP1A	2	2	2	2	2
CYP1B	1	1	1	1	1
CYP2A	2	3	4	3	3
CYP2B	6	8	5	6	1
CYP2C	3	15	7	15	4
CYP2D	2	2	7	5	1
CYP2E	1	0	1	1	1
CYP2F	1	1	1	1	1
CYP2G	0	1	1	1	0
CYP2J	2	6	7	4	1
CYP2R	1	0	1	1	1
CYP2S	1	1	1	1	1
CYP2T	0	1	1	1	0
CYP2U	0	0	1	1	1
CYP2W	0	1	1	1	1
CYP3A	4	4	8	6	4

Table 3-2: Pharmacokinetic parameters ($C_{\max}$ (ng·mL⁻¹), $T_{1/2}$ (min), AUC (μg·h·mL⁻¹), CL (mL·h⁻¹) for *in vivo* ACP exposure test.

Compound	Species	N	$C_{\max}$ (ng·mL ⁻¹)	$T_{1/2}$ (min)	AUC (μg·h·mL ⁻¹)	CL (mL·h ⁻¹)
ACP	Musk shrew	7	11940 ± 793	93 ± 9.1	1765 ± 130	0.68 ± 0.05
	Mouse	7	7947 ± 251	153 ± 12	1819 ± 97	0.66 ± 0.04
			*	*		
Des methyl-ACP	Musk shrew	7	7894 ± 460	447 ± 73	5452 ± 748	0.22 ± 0.03
	Mouse	7	8888 ± 1127	331 ± 29	4427 ± 671	0.28 ± 0.04
					*	

AUC: concentration-time area under the curve; $C_{\max}$: peak plasma concentration; T_{half} : plasma half-life; CL: clearance.

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

Data are shown as mean ± standard deviation (SD).

Table 3-3: Concentrations of cytochrome P450 (CYP) in liver microsomes (nmol·mg⁻¹ protein, n = 7).

Musk shrew	Mouse	Rat
0.55 ± 0.064 ^a	0.77 ± 0.019 ^b	0.99 ± 0.14 ^c

Different letter symbols among groups denote significant difference ($p < 0.05$, Steel-Dwass test).
Data are shown as mean ± SD.

Table 3-4: Michaelis–Menten kinetic parameters ($V_{\max}$ ($\text{pmol}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$), K_m (μM), $V_{\max}/K_m$ ($\mu\text{L}\cdot\text{min}^{-1}\cdot\text{mg}^{-1}$) for *in vitro* metabolic assay (n = 4).

Metabolite		Musk shrew	Mouse	Rat
des methyl-ACP	$V_{\max}/K_m$	12.1 ± 2.3^a	1.9 ± 0.37^b	3.4 ± 0.46^c
	$V_{\max}$	740 ± 61^b	231 ± 21^c	$1,282 \pm 100^a$
	K_m	61 ± 9^c	125 ± 19^b	388 ± 76^a

Different letter symbols among groups denote significant difference ($p < 0.05$, Steel-Dwass test).
Data are shown as mean $\pm$ SD.

Table 3-5: Percentages of produced amounts of dm-ACP compared to the control using CYP isoform-specific inhibitors and human liver microsome (%).

Inhibitor	Concentration of each inhibitor (μM)								
	0.001	0.01	0.1	0.5	1	5	10	50	100
Tranylcypromine (CYP2A6)	98.3	100.6	77.5	43.6	28.5	10.3	9.6	3.2	1.5
2-phenyl-1-2-(1-piperidiny) propane (CYP2B6)	93.5	100.3	97.6	91.3	100.8	103.5	99.3	101.6	82.5
Ticlopidine (CYP2C19)	104.3	98.8	100.3	110.3	105.8	111.4	104.7	103.2	102.4
Ketoconazole (CYP3A4)	101.2	104.3	102.1	89.5	101.3	98.5	97.8	76.4	58.6

Figure 3-1: Phylogenetic tree and synteny of *CYP1* genes using musk shrew, common shrew, mouse, rat, and human.



Figure 3-2: Phylogenetic tree and synteny of *CYP2* genes using musk shrew, common shrew, mouse, rat, and human.

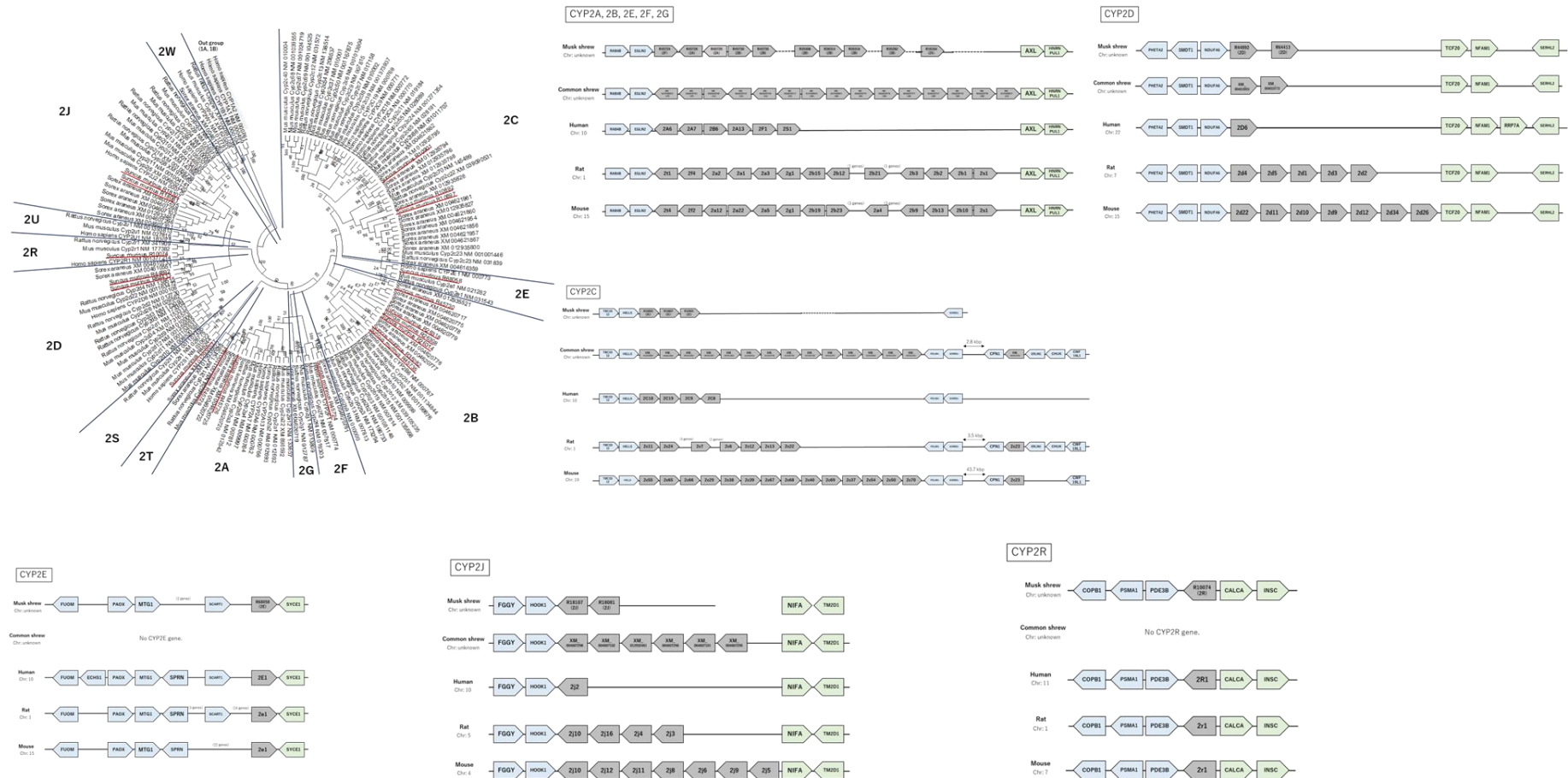


Figure 3-3: Phylogenetic tree and synteny of *CYP2* genes using musk shrew, common shrew, mouse, rat, and human.

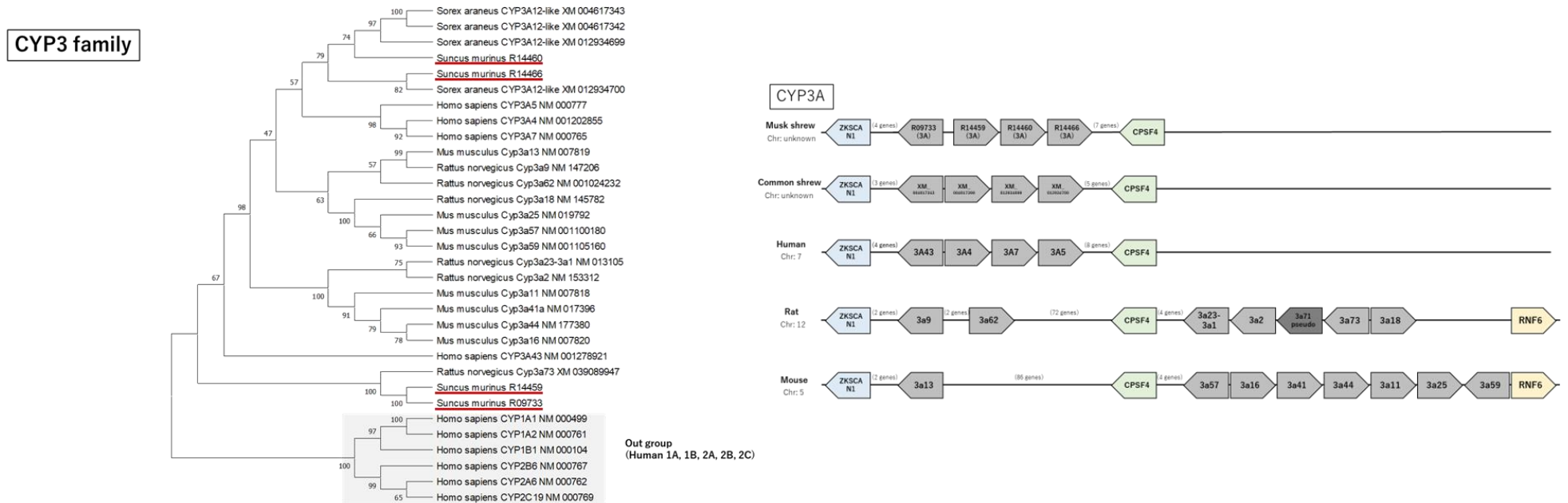


Figure 3-4: Transcripts per million (TPM) of *CYP* genes in the liver of musk shrew.

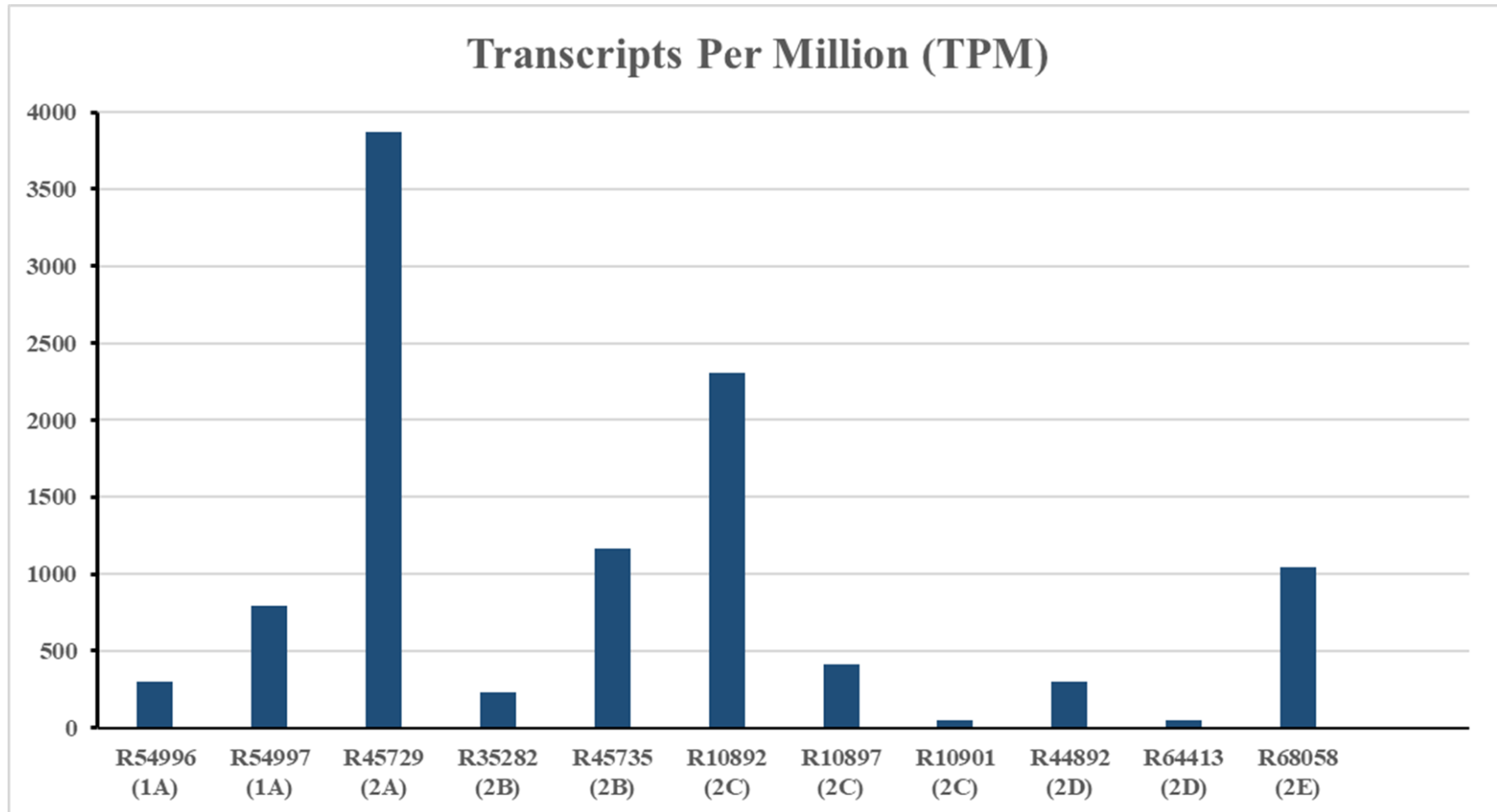


Figure 3-5: Blood concentration of acetamiprid and its metabolites in musk shrews and mice.

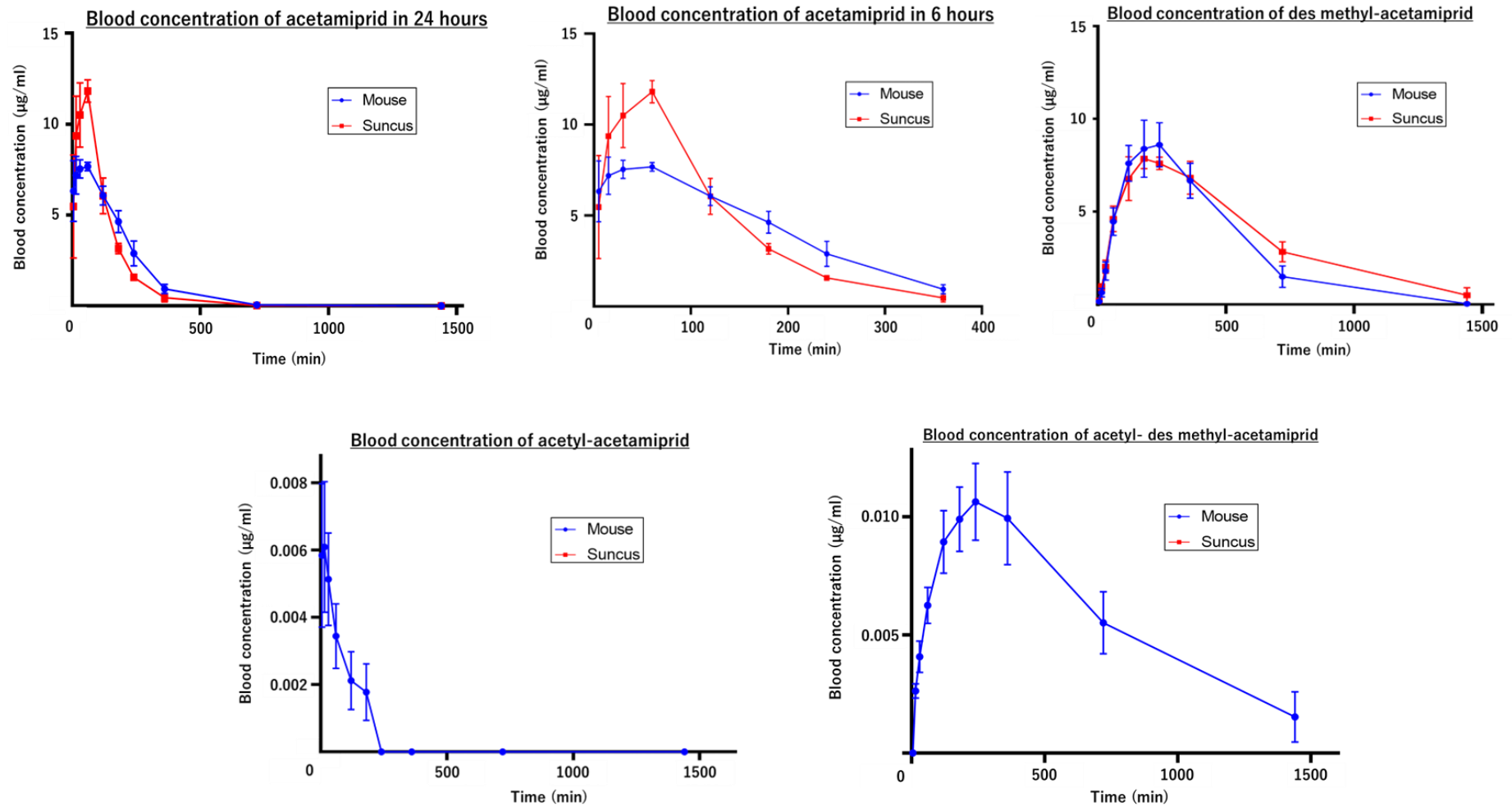
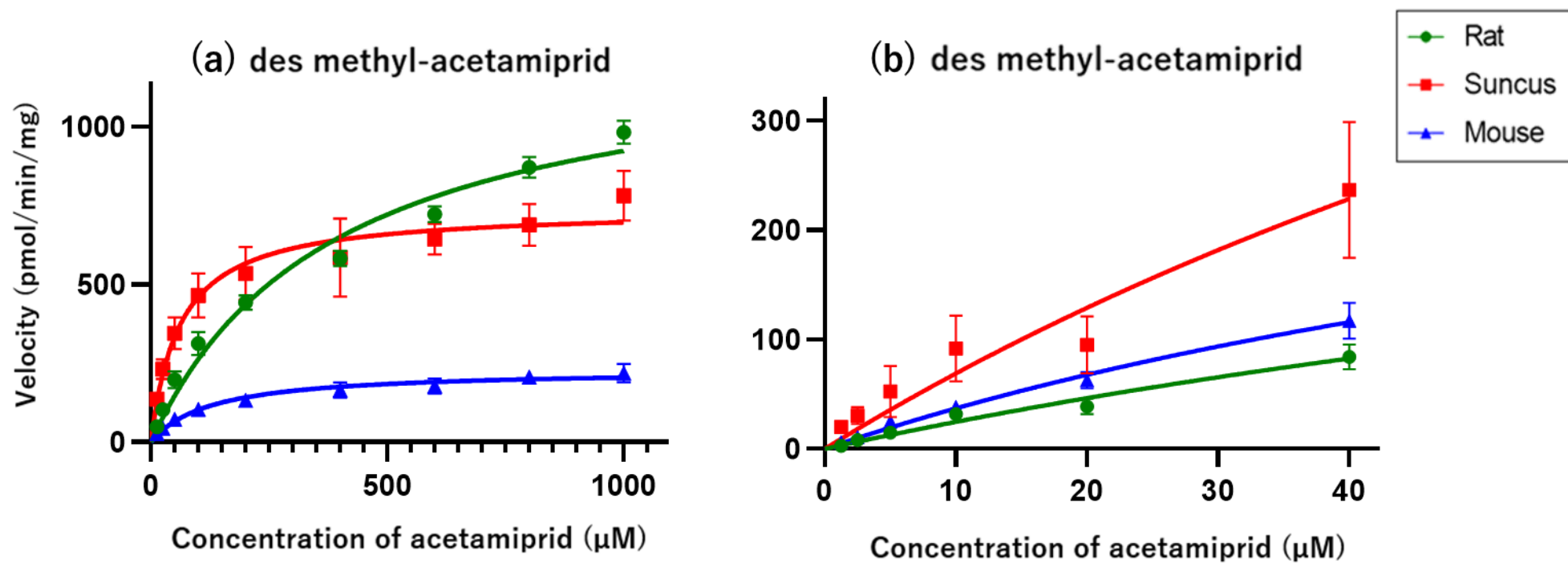


Figure 3-6: Enzyme kinetics for CYP-dependent metabolism of acetamiprid in musk shrews, mice, and rats.



Supplementary data

Table S3-1. CYP1 genes used in phylogenetic analysis.

Table S3-2. CYP2 genes used in phylogenetic analysis.

Table S3-3. CYP3 genes used in phylogenetic analysis.

Table S3-4. Detailed parameters for trimming and mapping in RNA-seq analysis.

Table S3-5: Recovery rate and matrix effect of acetamiprid and its metabolites in the blood.

Table S3-6: Details of animals used to obtain the liver.

Table S3-7: Parameters of LC-MS/MS for determination of acetamiprid and metabolites.

Table S3-1: CYP1 genes used in phylogenetic analysis.

Species	Scientific name	Gene name	Database	ID / Accession number
Musk Shrew	<i>Suncus murinus</i>	CYP1A1-like	1	R54996
		CYP1A2-like	1	R54997
		CYP1B1-like	1	R56407
European shrew	<i>Sorex araneus</i>	CYP1A1-like	2	XM_012933718
		CYP1A2	2	XM_004611739
		CYP1B1	2	XM_004616096
Rat	<i>Rattus norvegicus</i>	Cyp1a1	2	NM_012540
		Cyp1a2	2	NM_012541
		Cyp1b1	2	NM_012940
Mouse	<i>Mus musculus</i>	Cyp1a1	2	NM_001136059
		Cyp1a2	2	NM_009993
		Cyp1b1	2	NM_001364889
Human	<i>Homo sapiens</i>	CYP1A1	2	NM_000499
		CYP1A2	2	NM_000761
		CYP1B1	2	NM_000104

1: *Suncus murinus* Genome Project database (<https://suncus.nig.ac.jp/suncus/>)

2: National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>)

Table S3-2: CYP2 genes used in phylogenetic analysis.

Species	Scientific name	Gene name	Database	ID / Accession number
Musk Shrew	<i>Suncus murinus</i>	Uncharacterized	1	R45726
		Uncharacterized	1	R45729
		CYP2B5-like	1	R45730
		CYP2B11-like	1	R26308
		CYP2B11-like	1	R26314
		CYP2B11-like	1	R26318
		Uncharacterized	1	R35282
		CYP2B11-like	1	R45735
		Uncharacterized	1	R10074
		CYP2C20-like	1	R10892
		CYP2C21-like	1	R10901
		CYP2C27-like	1	R10897
		CYP2D14-like	1	R44892
		CYP2D17-like	1	R64413
		CYP2E1-like	1	R68058
		CYP2F5-like	1	R45724
		CYP2J2-like	1	R18081
		CYP2J2-like	1	R18107
		CYP2S1-like	1	R46244
European shrew	<i>Sorex araneus</i>	CYP2A13	2	XM_004620720
		CYP2A13-like	2	XM_004620722
		CYP2A13-like	2	XM_004620725
		CYP2B4-like	2	XM_012935521
		CYP2B11-like	2	XM_004620717
		CYP2B4-like	2	XM_004620775
		CYP2B11-like	2	XM_004620776
		CYP2B11-like	2	XM_004620777
		CYP2B4-like	2	XM_004620778
		CYP2B11-like	2	XM_004620779
		CYP2B4-like	2	XM_012935521
		CYP2C23-like	2	XM_004616359
		CYP2C19-like	2	XM_004621856
		CYP2C42-like	2	XM_004621860
		CYP2C21-like	2	XM_004621862
		CYP2C28-like	2	XM_004621867
		CYP2C18-like	2	XM_004621954
		CYP2C5-like	2	XM_004621957

		CYP2C5-like	2	XM_004621961
		CYP2C21-like	2	XM_012935794
		CYP2C21-like	2	XM_012935795
		CYP2C21-like	2	XM_012935796
		CYP2C21-like	2	XM_012935798
		CYP2C5-like	2	XM_012935800
		CYP2C19-like	2	XM_012935826
		CYP2C15-like	2	XM_012935827
		CYP2D14-like	2	XM_0046010551
		CYP2D14-like	2	XM_004610733
		CYP2F5	2	XM_004620781
		CYP2F2-like	2	XM_012935522
		CYP2G1	2	XM_004620719
		CYP2J2-like	2	XM_004607101
		CYP2J2-like	2	XM_004607102
		CYP2J2-like	2	XM_004607295
		CYP2J2-like	2	XM_004607296
		CYP2J2-like	2	XM_004607298
		CYP2J2-like	2	XM_012932461
		CYP2S1-like	2	XM_012935527
		CYP2W1	2	XM_004617263
Rat	<i>Rattus norvegicus</i>	Cyp2a1	2	NM_012692
		Cyp2a2	2	NM_012693
		Cyp2a3	2	NM_012542
		Cyp2b1	2	NM_001134844
		Cyp2b2	2	NM_001198676
		Cyp2b3	2	NM_173294
		Cyp2b12	2	XM_039105235
		Cyp2b15	2	NM_001135668
		Cyp2b21	2	NM_198733
		Cyp2c6	2	NM_001013904
		Cyp2c7	2	NM_017158
		Cyp2c11	2	NM_019184
		Cyp2c12	2	NM_031572
		Cyp2c13	2	NM_138514
		Cyp2c22	2	XM_039090531
		Cyp2c23	2	NM_031839
		Cyp2c24	2	NM_001271354
		Cyp2d1	2	NM_153313
		Cyp2d2	2	NM_012730
		Cyp2d3	2	NM_173093
		Cyp2d4	2	NM_138515

		Cyp2d5	2	NM_173304
		Cyp2e1	2	NM_031543
		Cyp2f4	2	NM_019303
		Cyp2g1	2	NM_012787
		Cyp2j3	2	NM_175766
		Cyp2j4	2	NM_023025
		Cyp2j10	2	NM_001134980
		Cyp2j13	2	XM_001058570
		Cyp2j16	2	XM_001058738
		Cyp2r1	2	XM_341909
		Cyp2s1	2	NM_001107495
		Cyp2t1	2	NM_134369
		Cyp2u1	2	NM_001393813
Mouse	<i>Mus musculus</i>	Cyp2a4	2	NM_009997
		Cyp2a5	2	NM_007812
		Cyp2a12	2	NM_133657
		Cyp2a22	2	NM_896592
		Cyp2b9	2	NM_010000
		Cyp2b10	2	NM_009999
		Cyp2b13	2	NM_007813
		Cyp2b19	2	NM_007814
		Cyp2b23	2	NM_001081148
		Cyp2c23	2	NM_001001446
		Cyp2c29	2	NM_007815
		Cyp2c37	2	NM_010001
		Cyp2c38	2	NM_010002
		Cyp2c39	2	NM_001373937
		Cyp2c40	2	NM_010004
		Cyp2c50	2	NM_001167875
		Cyp2c54	2	NM_206537
		Cyp2c55	2	NM_028089
		Cyp2c65	2	NM_028191
		Cyp2c66	2	NM_001011707
		Cyp2c67	2	NM_001024719
		Cyp2c68	2	NM_001039555
		Cyp2c69	2	NM_001104525
		Cyp2c70	2	NM_145499
		Cyp2d9	2	NM_010006
		Cyp2d10	2	XM_006520391
		Cyp2d11	2	NM_001104531
		Cyp2d12	2	NM_001355665
		Cyp2d22	2	NM_001163472

		Cyp2d26	2	NM_029562
		Cyp2d34	2	XM_017316570
		Cyp2e1	2	NM_021282
		Cyp2f2	2	NM_007817
		Cyp2g1	2	NM_013809
		Cyp2j5	2	XM_006502720
		Cyp2j6	2	NM_010008
		Cyp2j8	2	XM_974565
		Cyp2j9	2	NM_028979
		Cyp2j11	2	NM_001004141
		Cyp2j12	2	XM_143892
		Cyp2j13	2	NM_001346539
		Cyp2r1	2	NM_177382
		Cyp2s1	2	NM_028775
		Cyp2t4	2	NM_001100184
		Cyp2u1	2	NM_027816
		Cyp2w1	2	XM_620268
Human	<i>Homo sapiens</i>	CYP2A6	2	NM_000762
		CYP2A7	2	NM_000764
		CYP2A13	2	NM_000766
		CYP2B6	2	NM_000767
		CYP2C8	2	NM_000770
		CYP2C9	2	NM_000771
		CYP2C18	2	NM_000772
		CYP2C19	2	NM_000769
		CYP2D6	2	NM_000106
		CYP2E1	2	NM_000773
		CYP2F1	2	NM_000774
		CYP2J2	2	NM_000775
		CYP2R1	2	NM_001377214
		CYP2S1	2	NM_030622
		CYP2U1	2	NM_183075
		CYP2W1	2	NM_017781

1: Suncus murinus Genome Project database (<https://suncus.nig.ac.jp/suncus/>)

2: National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>)

Table S3-3: CYP3 genes used in phylogenetic analysis.

Species	Scientific name	Gene name	Database	ID / Accession number
Musk Shrew	<i>Suncus murinus</i>	CYP3A12-like	1	R09733
		CYP3A12-like	1	R14459
		CYP3A12-like	1	R14460
		CYP3A12-like	1	R14466
European shrew	<i>Sorex araneus</i>	CYP3A12-like	2	XM_004617342
		CYP3A12-like	2	XM_004617343
		CYP3A12-like	2	XM_012934699
		CYP3A13-like	2	XM_012934700
Rat	<i>Rattus norvegicus</i>	Cyp3a2	2	NM_153312
		Cyp3a9	2	NM_147206
		Cyp3a18	2	NM_145782
		Cyp3a23-3a1	2	NM_013105
		Cyp3a62	2	NM_001024232
		Cyp3a73	2	XM_039089947
Mouse	<i>Mus musculus</i>	Cyp3a11	2	NM_007818
		Cyp3a13	2	NM_007819
		Cyp3a16	2	NM_007820
		Cyp3a25	2	NM_019792
		Cyp3a41a	2	NM_017396
		Cyp3a44	2	NM_177380
		Cyp3a57	2	NM_001100180
		Cyp3a59	2	NM_001105160
Human	<i>Homo sapiens</i>	CYP3A4	2	NM_001202855
		CYP3A5	2	NM_000777
		CYP3A7	2	NM_000765
		CYP3A43	2	NM_001278921

1: Suncus murinus Genome Project database (<https://suncus.nig.ac.jp/suncus/>)

2: National Center for Biotechnology Information (NCBI) database (<https://www.ncbi.nlm.nih.gov/>)

Table S3-4: Detailed parameters for trimming and mapping in RNA-seq analysis.

Trimming (Trim Reads 2.6)		Mapping (RNA-seq Analysis 2.6)	
Trim using quality scores	Yes	Reference sequence	Genome sequence r131217
Quality limit	0.01	Gene track	gene_v1.0_Gene
Trim ambiguous nucleotides	Yes	mRNA track	gene_v1.0_mRNA
Maximum number of ambiguities	2	Use spike-in controls	No
Automatic read-through adapter trimming	Yes	Mismatch cost	2
Trim homopolymers from 5'	Yes	Insertion cost	3
Trim homopolymers from 3'	Yes	Deletion cost	3
polyA	Yes	Length fraction	0.8
polyC	Yes	Similarity fraction	0.8
polyG	Yes	Global alignment	No
polyT	Yes	Library type	Bulk
Remove 5' terminal nucleotides	No	Maximum number of hits for a read	10
Remove 3' terminal nucleotides	No	Count paired reads as two	No
Remove on first read	Yes	Ignore broken pairs	Yes
Remove on second read (for paired reads)	Yes	Expression value	TPM
Trim to a fixed length	No	Calculate expression for genes without transcripts	No
Maximum length	150	Minimum read count fusion gene table	5
Trim end	Trim from 3'-end		
Discard short reads	No		
Discard long reads	No		
Save discarded sequences	No		
Save broken pairs	No		

Table S3-5: Recovery rate and matrix effect of acetamiprid and its metabolites in the blood.

	ACP	dm-ACP	dc-ACP	dm-dc-ACP	N-ace-ACP	N-ace-dm-ACP
Recovery rate (%)	91.75	89.89	87.93	104.34	102.89	91.03
Matrix effect (%)	122.34	109.52	85.05	119.39	106.76	121.73

Table S3-6: Details of animals used to obtain the liver.

Species	Musk shrew	Mouse	Rat
Scientific name	<i>Procyon lotor</i>	<i>Mus Musculus</i>	<i>Rattus rattus</i>
Age	7 weeks	6 weeks	7 weeks
Sex	Male	Male	Male
Number of samples	4	4	4
Sample procurement	Tokyo Laboratory Animal Science (Tokyo, Japan)	CLEA (Tokyo, Japan)	Previous study (Khidkhan et al., 2021)

Table S3-7: Parameters of LC-MS/MS for determination of acetamiprid and metabolites.

Compound	Precursor ion	Product ion	CE	Polarity
ACP	223.08	126.0	24	Positive
		56.3	16	
ACP-d3	226.69	126.2	24	Positive
		59.2	16	
dm-ACP	209.06	125.8	20	Positive
		72.9	52	
dm-ACP-d3	212.21	126.2	24	Positive
		73.1	60	
dc-ACP	198.01	126.1	28	Positive
		73.1	56	
dm-dc-ACP	184.01	126.1	20	Positive
		73.0	60	
N-ace-ACP	199.01	126.1	20	Positive
		56.2	20	
N-ace-dm-ACP	185.01	126.0	20	Positive
		107.1	24	

RT: Retention time, CE: Collision energy

CHAPTER 4

Effects of Diazinon Exposure on Urinary Shedding of
Leptospira interrogans Serogroup Hebdomadis in Mice

Abstract:

Wild rodents are natural hosts of *Leptospira* spp. and are exposed to various pesticides, some of which are immunotoxic. Rodent urine is an important source of infection for humans and other animals. I evaluated the effects of pesticide exposure on *Leptospira* growth in mice. Diazinon, at doses of 0.2, 1, and 5 mg/kg/day, was orally administered continuously to mice infected with *Leptospira interrogans* serogroup Hebdomadis for 32 days. The numbers of *L. interrogans* in urine and kidney tissues were significantly lower in mice exposed to 5 mg/kg/day diazinon than in unexposed mice ($p < 0.05$). The urinary concentration of 2-isopropyl-6-methyl-4-pyrimidinol, the metabolite of diazinon, was comparable with the concentration at which viability of *L. interrogans* was decreased in *in vitro* assay, suggesting that it had toxic effects on *L. interrogans* in the proximal renal tubules. Diazinon exposure reinforced *Leptospira*-induced expression of inflammatory cytokine genes in kidney tissues, and an enhanced immune system might suppress *Leptospira* growth. These results suggest that diazinon exposure may not increase the risk of *Leptospira* transmission to humans through mice. This novel study evaluated the relationship between pesticide exposure and *Leptospira* infection in mice, and the results could be useful for risk assessment of leptospirosis.

Keywords

immunotoxicity; insecticide; pesticides; pollutant; infectious disease

Highlights

- Diazinon exposure at 5 mg/kg/day significantly reduced the number of *Leptospira* in urine and kidney tissue of mice compared to unexposed mice.
- Urinary concentrations of diazinon metabolites comparable to the *in vitro* effective concentration suggest toxic effects on *Leptospira* in the proximal renal tubules.
- Diazinon exposure induced elevated expression of inflammatory cytokine genes, potentially enhancing immune responses against *Leptospira*.
- The results suggest that diazinon exposure may not increase the risk of transmission of leptospirosis from mice to humans.

1. Introduction

Leptospirosis is a zoonotic disease that causes a variety of symptoms in humans and susceptible animal species (Bharti et al., 2003). *Leptospira* spp. colonize the kidney tissues and are excreted in the urine of reservoir animals such as rodents. Transmission occurs through direct contact with the urine of reservoir animals or indirectly via contact with the environment, such as soil and water, contaminated with their urine (Faine et al., 1999). Thus, the urine of wild rodents is an important infectious source of *Leptospira* spp. for humans or other animals.

Characterizing the urinary shedding of *Leptospira* spp. in rodents is a useful step in the risk assessment of human leptospirosis (Costa et al., 2015; Barragan et al., 2017). In natural environments, rodents are constantly exposed to various pesticides, including insecticides (Berny, 2007; Chi-Coyoc et al., 2016; Herrera et al., 2018). However, the effects of pesticide exposure on urinary shedding of *Leptospira* spp. by rodents have not been investigated.

Diazinon (DZN) is one of the most widely used insecticides; it is applied to various kinds of crops (e.g., corn, turnip, and onion) at doses of approximately 15–45 g/ha and is detected in environments globally (Aggarwal et al., 2013; Karyab et al., 2013; Abbasian et al., 2014). DZN elicits varying immune system effects on exposed animals depending on the exposure levels. At levels of 25–50 mg/kg/day, immunosuppression has been reported in mice; in the spleen, decreased weight, decreased cytokine gene expression levels of interleukin-2 (IL-2), IL-4, IL-10, and interferon- γ (IFN- γ), and suppressed humoral and cellular immune responses have been observed. In addition, decreased B cell concentrations in blood and suppression of lymphocyte proliferative responses have been reported (Neishabouri et al., 2004;

Alluwaimi & Hussein, 2007). In contrast, increased mRNA expression levels of cytokine genes, including tumor necrosis factor- α (TNF- α) and IL-10 in blood, have been observed in response to lower concentrations (1.5–6.5 mg/kg/day) of DZN exposure; this may indicate enhanced immune responses (Maliji et al., 2014; Ali et al., 2020). These cytokines are involved in the immune response to leptospirosis, and the DZN-induced effects may affect the growth of *Leptospira* spp. in infected mice (Marinho et al., 2005; Monahan et al., 2009; Bavia et al., 2019).

At more than 30 mg/kg/day, DZN causes histological changes in the kidneys, including degeneration and loss of glomerular integrity (Cakici & Akat, 2013). If histological changes also occur at the concentration equivalent to the environmental exposure level, urinary shedding of *Leptospira* spp. could be affected. In addition, DZN could show lethal effects on *Leptospira* spp. in the kidney, although there is no report regarding DZN-induced toxicities for the bacteria.

The purpose of this study was to evaluate the effect of DZN exposure on urinary shedding of *Leptospira* spp. in mice, considering the environmental situation in which rodents are exposed to both *Leptospira* spp. and DZN. The number of *L. interrogans* in urine and kidney tissue was investigated following oral administration of DZN to mice infected with *L. interrogans*. Additionally, cytokine gene expression levels and histological changes in the kidneys were determined following exposure.

2. Materials and Methods

2.1. Culture Conditions of L. interrogans

Leptospira interrogans serogroup Hebdomadis strain OP84 (Fujita et al., 2015) was used in this study. The strain was stored in Korthof's medium containing 10%

glycerol at -80°C until each experiment. For the *in vitro* viability assay, the strain was thawed and cultured in liquid Korthof's medium at room temperature at first passage and then cultured in Ellinghausen–McCullough–Johnson–Harris (EMJH) medium after second passage. *In vitro* passage was conducted fewer than three times before the assay. For *in vivo* infection, the strain was thawed and passaged twice using liquid Korthof's medium and then inoculated into mice.

2.2. *In vitro* Viability Assay of *L. interrogans*

The lethal dose of DZN (Fujifilm, Tokyo, Japan) and its major metabolite, 2-isopropyl-6-methyl-4-pyrimidinol (IMP) (Santa Cruz Biotechnology, Santa Cruz, CA, USA), were investigated according to the reported method (Mouville & Benaroudj, 2020). In a round bottom 96-well plate, 180 μL of 5×10^6 cell/mL 7 day-cultured OP84 strain was inoculated into each well. The number of leptospiral cells was determined using a Thoma cell counting chamber (Yazawa Science, Tokyo, Japan) and dark field microscopy. Then, 20 μL of serial two-fold diluted DZN, IMP, or ampicillin (as a positive control) in EMJH medium containing 1% methanol ranging from 0.03 to 500 ppm was added. Each plate also included a negative control (EMJH containing 1% methanol without DZN or IMP). After 3 days of incubation at room temperature, 20 μL of 10-fold diluted AlamarBlue Cell Viability Reagent (Thermo Fisher Scientific, Waltham, MA, USA) was added to each well. After an additional 2 days of incubation, absorbance at 570 nm was measured using a spectrometer (Multiskan GO, Thermo Fisher Scientific). Cell viability was represented as a ratio with the negative control wells. Assays were performed in triplicate.

To confirm the stability of DZN and IMP in EMJH medium during the assay, wells containing *L. interrogans* and four concentrations of DZN or IMP at 1.95, 7.8, 15.6, and 250 ppm were prepared; 100 μ L of medium was collected before and after the assay and the concentration of DZN and IMP was measured as described below (Section 2.10).

2.3. *Leptospira* Infection and Exposure to DZN

Seven-week-old male ICR mice ($n = 72$) were used for the *in vivo* experiments. Mice were purchased from CLEA (Tokyo, Japan) and kept at 20–23°C under a 12 h/12 h light/dark cycle. Water and food (CE-2; CLEA) were given ad libitum. Experiments were performed after a 2 week acclimation period.

Mice were categorized into eight groups (each group: $n = 9$): control; low, medium, and high-dose DZN exposure (LD, MD, HD, respectively); *Leptospira* infection without DZN exposure (Lepto); and *Leptospira* infection with low, medium, and high-dose DZN exposure (Lepto + LD, Lepto + MD, and Lepto + HD, respectively).

The mice in all four groups infected with *Leptospira* were intraperitoneally inoculated with 3×10^6 cells of strain OP84 in the log phase suspended in 150 μ L of Korthof's medium and then kept in mesh cages for approximately 1 h. The number of leptospiral cells was determined as mentioned above. The same volume of Korthof's medium was intraperitoneally inoculated to all other groups.

Following inoculation, excreted urine was collected in 1.5 mL tubes at 6, 8, 10, 13, 16, 18, 21, 24, 27, and 31 days post infection (dpi). Urine samples were kept at –80°C until analysis. For DZN exposure, at 31 dpi, DZN was dissolved in the drinking water of all the groups except the control and Lepto groups. DZN was then provided

orally to the mice ad libitum. The amount of water that the mice drank was estimated as 5 mL/day (Murai et al., 2010). The dose of DZN used was 0.2, 1.0, and 5.0 mg/kg/day for low, medium, and high doses, respectively. These doses were set based on the estimated maximum exposure dose (6.13 mg/kg/day) as defined by the deposit of DZN at the recommended dosage on grapes (9.04 mg/kg) (Torabi & Talebi, 2013). With the assumption that mice depended entirely on grapes contaminated with 9.04 mg/kg of DZN for their diet, an estimated exposure dose can be calculated as 6.13 mg/kg/day using the parameters of 25 g of body weight (Vellers et al., 2017), 0.59 kcal/g of energy in grapes (Food Composition Database provided by Ministry of Education, Culture, Sports, Science and Technology (Public Database of National Institute of Environmental Studies. Available online: <https://fooddb.mext.go.jp>), and 10 kcal/day of energy requirements for mice (Vellers et al., 2017). DZN exposure was continued until 63 dpi.

During the exposure period, urine samples of *Leptospira*-infected mice were collected at 34, 37, 41, 45, 48, 51, 53, 56, and 63 dpi, as described above. All animals survived without any abnormalities during experiments. At 63 dpi, mice were sacrificed by CO₂ inhalation. After euthanasia, kidney tissues were collected immediately. A fraction of the right renal cortex was stored at -20°C in 2 mL of RNAlater (Thermo Fisher Scientific) and stored at 4°C overnight until DNA or RNA extraction. Other fractions of the right renal cortex were stored at -20°C for quantification of DZN and IMP residue levels. Left kidneys were stored in 10% of formalin for histological examination.

All animal care and experimental procedures were performed in accordance with the Guidelines of the Association for Assessment and Accreditation of Laboratory

Animal Care International (AAALAC) and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Approval number: 22-23).

2.4. DNA Extraction from Kidney and Urine Samples

Genomic DNA was extracted from 25 mg of the renal cortex and 20 μ L of urine of *Leptospira*-inoculated mice using QIAamp DNA Mini Kit (Qiagen, Victoria, Australia). The concentrations of extracted DNA from the renal cortex were determined using Nano Drop Spectrometer ND-1000 (Thermo Scientific, Rockford, IL, USA) and adjusted to 50 ng/ μ L with double distilled water (DDW) for quantitative PCR. DNA from urine samples was applied to quantitative PCR without concentration adjustment, as described by Soupé-Gilbert et al. (Soupé-Gilbert et al., 2017).

2.5. RNA Extraction and cDNA Synthesis from Kidney

Total RNA was extracted from approximately 20 mg of the renal cortex using RNA Basic Kit (FastGene, Tokyo, Japan). The concentration of extracted RNA was determined using Nano Drop Spectrometer ND-1000. cDNA synthesis with 500 ng of extracted RNA was conducted using ReverTra Ace qPCR RT Master Mix with gDNA remover (Toyobo, Osaka, Japan). cDNA was diluted to 50 ng/ μ L with DDW for absolute quantitative PCR.

2.6. Real-Time PCR

Real-time PCR was performed using QuantStudio 12K Flex Real-Time PCR System and software version 1.2.2 (Thermo Fisher Scientific). PCR reactions were

conducted in 10- μ L volumes containing Fast SYBR Green Master Mix (Thermo Fisher Scientific) and 300 nM forward and reverse primers. Sequence information of the primers used in this study are listed in Table S4-1. The PCR condition was as follows: initial denaturation at 95°C for 20 s, 40 cycles of denaturation and amplification of target sequences at 95°C for 1 s, and 60°C for 20 s, followed by melting curve analysis to confirm the specificity of amplified products. The efficiency of each primer set was calculated using eightfold dilution of the template DNA, and all primer sets showed acceptable efficiencies within 95–105% (Table S4-1).

2.7. Quantification of *Leptospiral* DNA in Kidney and Urine

Leptospiral DNA in mouse kidney and urine samples was quantified by absolute quantification of 16S rRNA gene. A standard curve was obtained using serially diluted genomic DNA extracted from the OP84 strain. The mouse ribosomal protein L18 (RPL18) gene was simultaneously quantified as a housekeeping gene to validate the quantity and quality of DNA samples. Quantity of *leptospiral* DNA in kidney and urine samples was shown as *leptospiral* genome equivalent per μ g genome and per ml of urine, respectively, considering a genome size of 4.6 Mbp corresponding to *L. interrogans* Fiocruz L1-130 (Nascimento et al., 2004).

2.8. Quantification of Expression Levels of Cytokine Genes in Kidney Tissues

mRNA expression levels of cytokine genes including IL-1 β , IL-2, IL-4, IL-6, TNF- α , TNF- β , IFN- γ , transforming growth factor- β (TGF- β), macrophage inflammatory protein (MIP-1), and IFN- γ -induced protein-10 (IP-10) were quantified.

Plasmids were constructed to make standard curves for quantifying cDNA copy numbers of targeted cytokine genes. The cytokine genes mentioned above and internal standard candidate genes, including ribosomal protein L 18 (RPL18), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), β -actin, and β -2-microglobulin gene, were amplified from the cDNA libraries prepared above by PCR using SappireAmp Fast PCR Master Mix (Takara, Shiga, Japan). PCR was conducted at 94°C for 1 min, followed by 35 cycles of amplification of target sequences at 98°C for 5 s, 60°C for 5 s, and 72°C for 5 s, and a final extension at 72°C for 1 min. The PCR products were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) and were cloned into pCR2.1-TOPO vector using a TOPO TA Cloning Kit (Thermo Fisher Scientific).

RPL18 gene was used as an internal control because it showed the most stable expression levels between control mice and DZN exposed or *Leptospira*-infected mice among the internal standard candidate genes described above. Expression levels of cytokine genes in each mouse group are represented as a ratio to that of the control group. Ct 35 was applied as the cut-off value for real time PCR analysis.

2.9. Histological Examination of Kidney Tissue

Histological examination was performed by Sapporo General Pathology Laboratory (Sapporo, Japan). Kidney tissues ($n = 3$ for each group, total 24 samples) fixed in 10% buffered formalin were embedded in paraffin. The embedded kidney tissues were sectioned and stained with hematoxylin and eosin. Renal cortex tissue was observed using an optical microscope.

2.10. Extraction and Quantification of DZN and IMP in Kidney Tissues, Urine, and EMJH Medium

Five milligrams of renal cortex were added to 500 μL of 1% formic acid in acetonitrile and homogenized with horizontal shaking at a frequency of 30/s for 1 min using Tissue Lyser (QIAGEN, Hilden, Germany). Homogenized samples were centrifuged at $10,000\times g$ for 5 min and the supernatant was collected. MonoSpin Phospholipid (GL Sciences, Tokyo, Japan) was preconditioned with 200 μL of 1% formic acid in acetonitrile by centrifugation at $3000\times g$ for 5 min. 100 μL of the collected supernatant was passed through the MonoSpin Phospholipid by centrifugation at $3000\times g$ for 5 min, and 80 μL of the elute was added to 240 μL of 0.1% formic acid in DDW and transferred to a plastic vial for quantitative analysis using liquid chromatography-mass-spectrometry (LC/MS/MS).

In brief, 5 μL of urine sample or EMJH medium was added to 500 μL of 1% formic acid in acetonitrile and centrifuged at $10,000\times g$ for 5 min. Then, 100 μL of the supernatant was added to 300 μL of 0.1% formic acid in DDW and transferred to vials for LC/MS/MS analysis.

LC/MS/MS (6495 triple quad LC/MS; Agilent Technologies, Santa Clara, CA, USA) equipped with an InterSustainSwift C18 column (2.1×50 mm, $\phi 1.9$ μm , GL Sciences, Tokyo, Japan) was used to quantify DZN and IMP. In the analysis using high performance LC (HPLC), solvent A was 0.1% (v/v) formic acid and 5 mM ammonium formate in DDW, and solvent B comprised 0.1% (v/v) formic acid in acetonitrile. The following gradient was applied: $t = 0$ –0.2 min, 5% solvent B; $t = 4$ min, 95% solvent B; $t = 4$ –5 min, 100% solvent B. 50°C of the column oven temperature, 0.6 mL/min of flow rate, and 5 μL of injection volume were used. Multiple-reaction monitoring

(MRM) for mass spectrometry was programmed as shown in Table S4-2. Calibration curves were applied for DZN and IMP using final concentrations of 0.5, 1, 5, 10, 25, and 50 ng/mL. The recovery rate and matrix effects were calculated using kidney and urine samples from control group mice and EMJH medium ($n = 5$), as shown in Table S4-3. The recovery rates showed small variations, and thus the concentrations of DZN and IMP were calculated by dividing the measured values by the recovery rate.

2.11. Statistical Analysis

Statistical analyses were conducted using the JMP® Pro 16 software (SAS Institute, Cary, NC, USA). The Shapiro–Wilk test and Levene’s test were performed to evaluate parametric distribution and variance homogeneity of the data, respectively. The Steel–Dwass test was performed to compare the urinary shedding numbers of *L. interrogans*, the number of *L. interrogans* colonies in kidney tissues, and the expression levels of cytokine genes among each group. The Wilcoxon test was applied for the comparison of *L. interrogans* viability in *in vitro* assays between the control and each exposure concentration. A p -value < 0.05 was considered statistically significant for all analyses.

3. Results

3.1. Lethal Concentrations of DZN and IMP for *L. interrogans* on *In vitro* Assay

Figure 4-1 shows the viability of the *L. interrogans* strain exposed to ampicillin, DZN, and IMP *in vitro*. In the ampicillin assay, the viability of the strain was significantly decreased when compared to the control at a concentration of 0.03 ppm ($51.1 \pm 6.6\%$, $p = 0.007$) and higher (Figure 4-1A). Viability was significantly lower

than control above a DZN concentration of 250 ppm ($81.1 \pm 3.4\%$, $p = 0.032$) and 16 ppm for IMP ($91.1 \pm 1.3\%$, $p = 0.041$) (Figure 4-1B, C). DZN and IMP were stable during the assays (ratio of DZN and IMP concentrations 5 days after the assay to that before assay were 93.5–112.7% and 92.7–107.9%, respectively; Table S4-4).

3.2. Urinary Shedding of *L. interrogans* in Mice Exposed to DZN

The concentration of *L. interrogans* shed in urine increased until 21 dpi and reached a plateau at approximately 2×10^7 genome equivalents/mL (Figure 4-2). After DZN exposure, there was no significant differences in urinary *L. interrogans* among all groups until 53 dpi (Figure 4-3). However, urinary *L. interrogans* in Lepto + HD was significantly lower than other groups at 56 dpi (Lepto + HD: $0.58 \pm 0.53 \times 10^7$ /mL vs. control: $1.89 \pm 0.25 \times 10^7$ /mL, $p = 0.012$; vs. Lepto + LD: $2.48 \pm 0.23 \times 10^7$ /mL, $p = 0.021$; vs. Lepto + MD: $1.89 \pm 0.20 \times 10^7$ /mL, $p = 0.010$) and at 63 dpi (Lepto + HD: $0.77 \pm 0.35 \times 10^7$ /mL vs. control: $1.99 \pm 0.69 \times 10^7$ /mL, $p = 0.006$; vs. Lepto + LD: $2.22 \pm 1.07 \times 10^7$ /mL, $p = 0.019$; vs. Lepto + MD: $1.79 \pm 1.09 \times 10^7$ /mL, $p = 0.043$).

3.3. *L. interrogans* in Kidney Tissues of Mice Exposed to DZN

Kidney *L. interrogans* levels at 63 dpi (32 days after DZN exposure) are shown in Figure 4-4. The levels in the Lepto + HD group was significantly lower than those of the control and Lepto + LD groups (Lepto + HD: $0.62 \pm 0.44 \times 10^4$ /μg vs. control: $1.54 \pm 0.62 \times 10^4$ /μg, $p = 0.019$; vs. Lepto + LD: $1.43 \pm 0.88 \times 10^4$ /μg, $p = 0.032$). There was no statistical difference between Lepto + LD and Lepto + MD groups ($1.27 \pm 0.59 \times 10^4$ /μg, $p = 0.33$).

3.4. Concentrations of DZN and IMP in Kidney Tissue and Urine of Exposed Mice

The concentrations of DZN and IMP in the kidney tissue and urine of mice at 63 dpi (32 days after DZN exposure) increased depending on the orally exposed DZN dose (Table 4-1). Even in the HD group, DZN and IMP concentrations (2.21 ± 0.56 and 0.66 ± 0.12 ppm, respectively) in kidney tissue were much lower than the effective concentration of *in vitro* assays (250 and 16 ppm for DZN and IMP, respectively). In urine samples, the concentration of IMP in the HD group was near the effective concentration of the *in vitro* assay (12.51 ± 2.1 ppm); this was not seen for DZN (0.56 ± 0.27 ppm).

3.5. Pathological Characteristics of Kidney Tissues of *Leptospira*-Infected Mice

Exposed to DZN

Figure 4-5 shows the histological images of kidney tissues in each group. No consistent pathological changes were observed in all groups.

3.6. mRNA Expression Levels of Cytokine Genes in Kidney Tissue of DZN-Exposed Mice and *Leptospira*-Infected Mice Exposed to DZN

The expression levels of cytokine genes in kidney tissue of *Leptospira*-infected mice with or without DZN are shown in Figure 4-6. Several cytokine gene expression levels were elevated by DZN exposure: The expression level of IFN- γ in the LD group was significantly higher than that of the control group ($p < 0.05$). Between the control and MD groups, significantly higher expression levels were observed in TNF- α , IFN- γ , and MIP-1 ($p < 0.05$). Increased cytokine gene expression was observed for IL-1 β , IL-6,

TNF- α , TNF- β , IFN- γ , TGF- β , and MIP-1 in the HD group when compared to the control group ($p < 0.05$).

Elevated expression levels by *Leptospira* infection were also observed in several cytokine genes. The expression levels in the Lepto group were significantly higher than those in the control group for IL-1 β , IL-6, TNF- α , TNF- β , IFN- γ , and MIP-1 ($p < 0.05$). Furthermore, DZN exposure to *Leptospira*-infected mice additively increased the expression of these cytokine genes. Compared to the Lepto group, significantly elevated gene expressions were observed in the Lepto + MD group for IL-1 β and IL-2 ($p < 0.05$). The expression levels in the Lepto + HD group were significantly higher than that of the Lepto group for IL-1 β , IL-2, IL-6, TNF- α , TGF- β , MIP-1, and IP-10 ($p < 0.05$). There was no significant difference in expression levels of any cytokine genes between the Lepto + LD and Lepto groups.

Total RNA was extracted from the renal cortex and used for cDNA synthesis. Absolute quantification of gene expression levels of cytokines including IL-1 β , IL-2, IL-4, IL-6, TNF- α , TNF- β , IFN- γ , TGF- β , MIP-1, and IP-10 were performed (Mean $\pm$ SD, $n = 9$). Expression levels of targeted cytokine genes in each mouse group are represented as the ratio to that of the control group. Several cytokine gene expression levels were elevated as a result of DZN exposure. Elevated expression levels by *Leptospira* infection were also observed in several cytokine genes. Furthermore, DZN exposure to *Leptospira*-infected mice additively increased the expression of these cytokine genes.

4. Discussion

4.1. Effect of DZN Exposure on Leptospira Proliferation in Kidney Tissue

Leptospira spp. colonize and proliferate in the kidneys of reservoir animals, and some of the bacteria are excreted in the urine (Faine et al., 1999). A positive correlation between leptospiral numbers in urine and kidney tissue was confirmed in wild rats (Costa et al., 2015). In this study, the decreased number of *L. interrogans* in the kidney tissue following DZN exposure was likely to lead to a reduced number of bacteria shed in the urine. Although it has been reported that exposure to DZN at a concentration of 30 mg/kg caused histological changes to the kidney tissue of mice (Cakici & Akat, 2013), this was not observed at the lower concentrations investigated in the present study. Thus, it is unlikely that *Leptospira* proliferation and/or excretion was influenced by pathological changes to renal tissue.

In the proximal renal tubules where *Leptospira* spp. colonize, approximately 70% of filtered water by glomerulus is reabsorbed (Curthoys & Moe, 2014), and the concentrations of some chemicals increase (Van Vleet & Schnellmann, 2003). Therefore, the concentration of IMP in the lumen of proximal tubules might be higher than that in blood and comparable with urinary concentration. *Leptospira* regularly reside in the lumen of the proximal tubules (Athanzio et al., 2008; Santos et al., 2015) and might be exposed to IMP in the lumen. Urinary concentrations of IMP in the present study were near the effective concentrations obtained by *in vitro* viability assay. Thus, this suggests that colonized *L. interrogans* in the proximal renal tubules might be exposed to the effective concentration of IMP for longer periods than the *in vitro* viability assay (*in vitro*: 5 days, *in vivo*: 35 days), leading to suppression of bacterial proliferation.

The expression levels of most of the cytokine genes examined increased depending on the exposure concentrations of DZN. These results are consistent with

previous reports that mRNA expression levels of some cytokine genes were upregulated with DZN exposure below 6.5 mg/kg/day (Maliji et al., 2014; Ali et al., 2020). Moreover, the expression levels of cytokines, including IL-1 β , IL-2, IL-6, TNF- α , TGF- β , MIP-1, and IP-10, in the kidney tissues were additively elevated by DZN exposure and *Leptospira* infection. IL-1 β , IL-2, TNF- α , and MIP-1 are involved in activation of inflammatory cells, including B cells, T cells, macrophages, and natural killer cells (Schall & Bacon, 1994; Oberholzer et al., 2000). IL-6 acts as a potent stimulant for B-cell proliferation and antibody production (Muraguchi et al., 1988). MIP-1 and IP-10 promote the migration of leukocytes (Schall & Bacon, 1994). The additive induction of cytokines may lead to enhanced immune system function for effective defense against *L. interrogans*. To verify this hypothesis, the interval between elevated cytokine gene levels and decreased *L. interrogans* in kidney tissue needs to be confirmed. Furthermore, it should be noted that other possible DZN-induced factors, such as oxidative stress (Shah & Iqbal, 2010) and DNA damage (Ali et al., 2020), might affect the proliferation of *L. interrogans*; this must also be explored in the future.

In this study, mice were infected with *L. interrogans* followed by DZN exposure based on vertical transmission. A significant proportion of wild rodents are infected with *Leptospira* spp. from their parents before leaving the nest (Minter et al., 2017), and wild rodents are thought to be exposed to pesticides after *Leptospira* infection. On the contrary, horizontal transmission, where rodents become infected with *Leptospira* spp. after leaving the nest, can also occur (Minter et al., 2017). In this situation, DZN exposure can occur before mice get infected with *L. interrogans*, and DZN might affect not only the proliferation but also the initial colonization of *Leptospira* spp. in the

kidneys. The effects of DZN exposure on horizontal transmission of *L. interrogans* should be investigated in the future.

4.2. Influence of DZN Exposure on the Infectious Risk of Leptospirosis in Human

The urine of reservoir animals is the primary infection source of *Leptospira* spp. for humans and a key factor in the environmental cycling of *Leptospira* spp. (Faine et al., 1999). In a previous study, the infection risk of *Leptospira* spp. for humans was evaluated by applying models using parameters such as climate factor, serotypes of *Leptospira* spp., population densities, and urine volume of host animals (Costa et al., 2015). The infection risk of leptospirosis for humans is increased as the number of *Leptospira* spp. shed in urine becomes higher (Costa et al., 2015; Barragan et al., 2017). Considering the decreased urinary shedding of *L. interrogans* due to DZN exposure in this study, it might be suggested that environmental DZN may not increase the risk of leptospirosis for people living in surrounding areas. As a limitation, it should be noted that various other chemicals that have the potential for immunotoxicities are present in the environments, but I can evaluate only diazinon-induced effects on urinary *Leptospira* shedding from this study.

5. Conclusions

This is the first study to examine the effects of chemical exposure on dynamics of a bacterial pathogen in reservoir animals using diazinon and *L. interrogans* as a model. Wild animals are generally important carriers of various zoonotic pathogens (Karesh et al., 2012) and are exposed to a variety of environmental pollutants with immunotoxic potential, including pesticides, heavy metals, and drugs (Boyd, 2010;

Corsini et al., 2013; Suzuki et al., 2020). In the future, it will be necessary to investigate the effects of various environmental pollutants on infectious diseases other than leptospirosis.

Table 4-1: Concentrations of DZN and IMP in kidney tissues and urine.

Compound	Exposure Dose	Measured Concentration (ppm) *	
		Kidney	Urine
DZN	0.2 mg/kg/day	0.25 ± 0.10	0.07 ± 0.03
	1.0 mg/kg/day	1.03 ± 0.34	0.14 ± 0.07
	5.0 mg/kg/day	2.21 ± 0.56	0.56 ± 0.27
IMP	0.2 mg/kg/day	0.23 ± 0.05	0.81 ± 0.19
	1.0 mg/kg/day	0.32 ± 0.09	2.57 ± 0.80
	5.0 mg/kg/day	0.66 ± 0.12	12.51 ± 2.1

* Average ± SD; DZN: Diazinon, IMP: isopropyl-6-methyl-4-pyrimidinol,

SD: Standard deviation.

Figure 4-1. Viability of *L. interrogans* in *in vitro* assay using ampicillin (A), diazinon (B), and IMP (C) (Mean $\pm$ SE, n = 3).

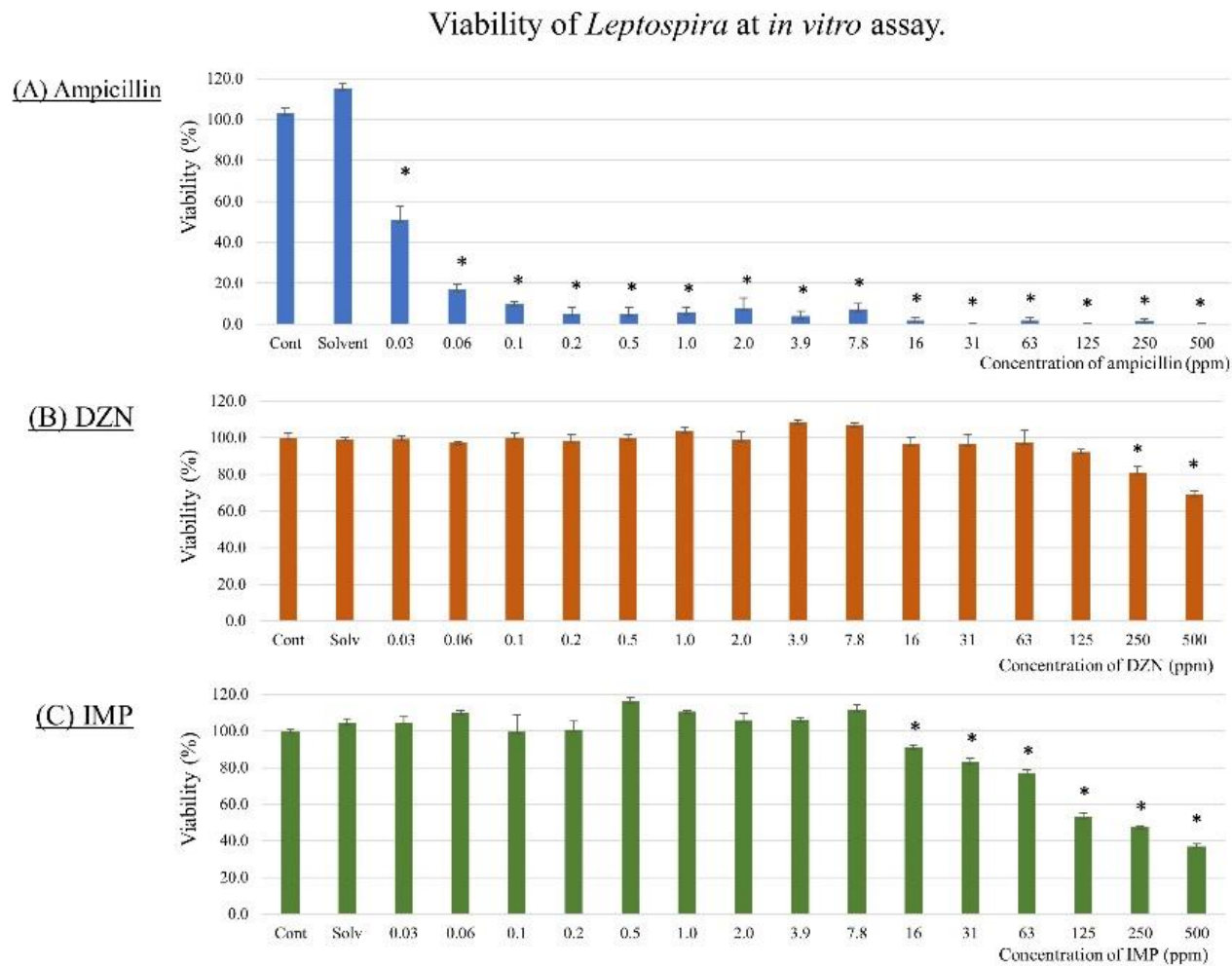


Figure 4-2. Urine *L. interrogans* quantification before diazinon exposure.

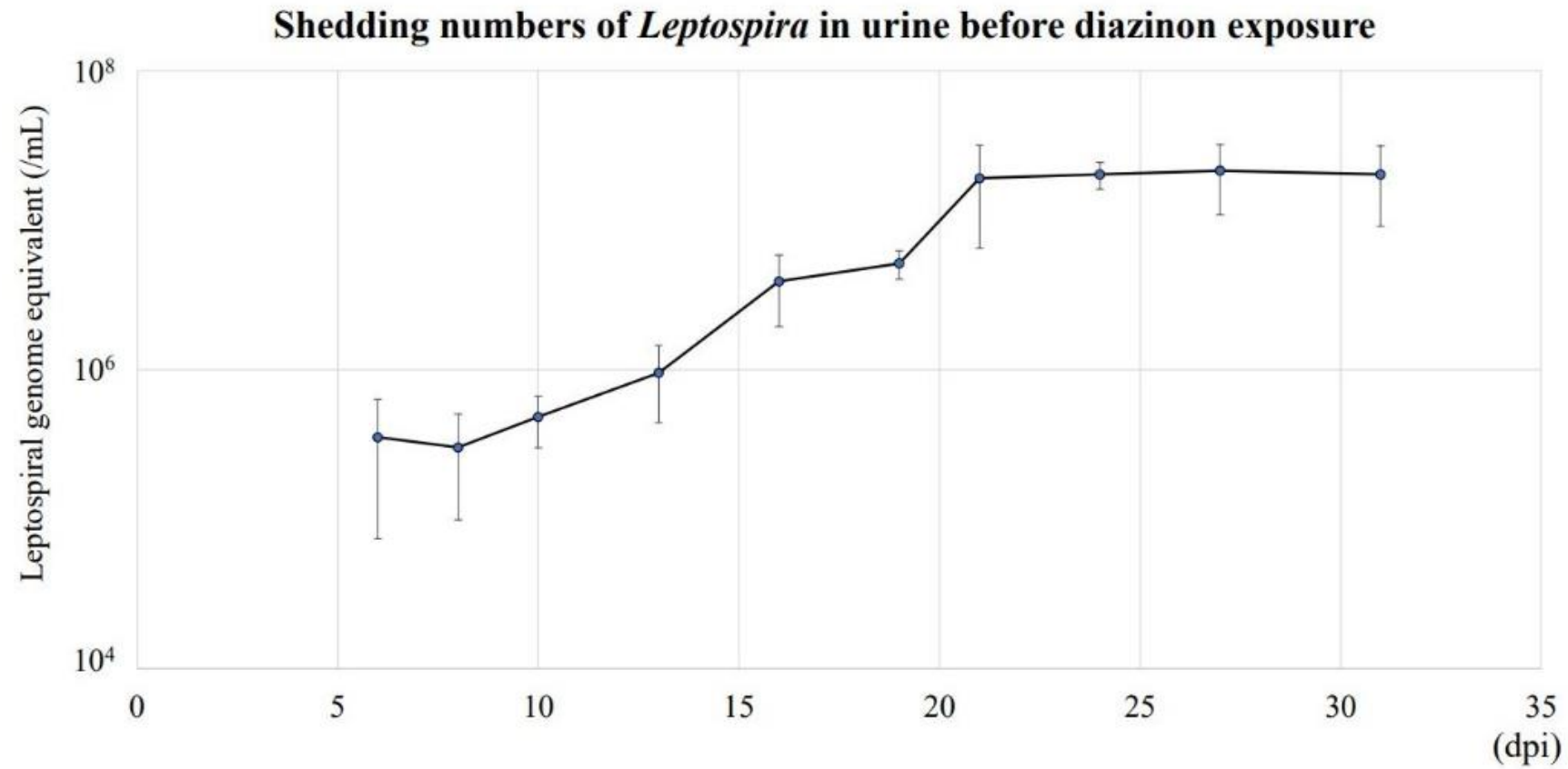


Figure 4-3. Urine *L. interrogans* quantification after diazinon exposure.

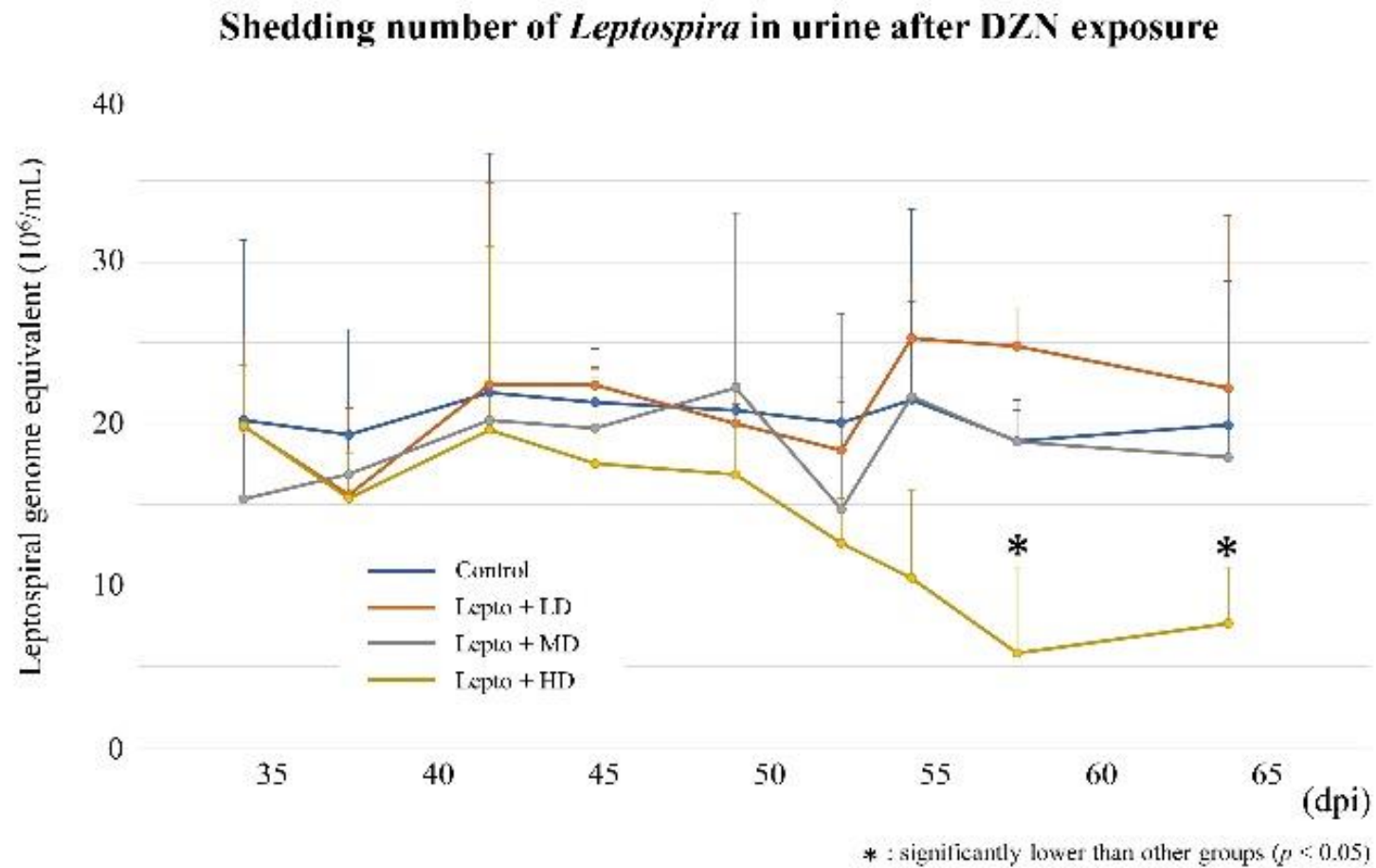


Figure 4-4. Quantification of *L. interrogans* in kidney tissues.

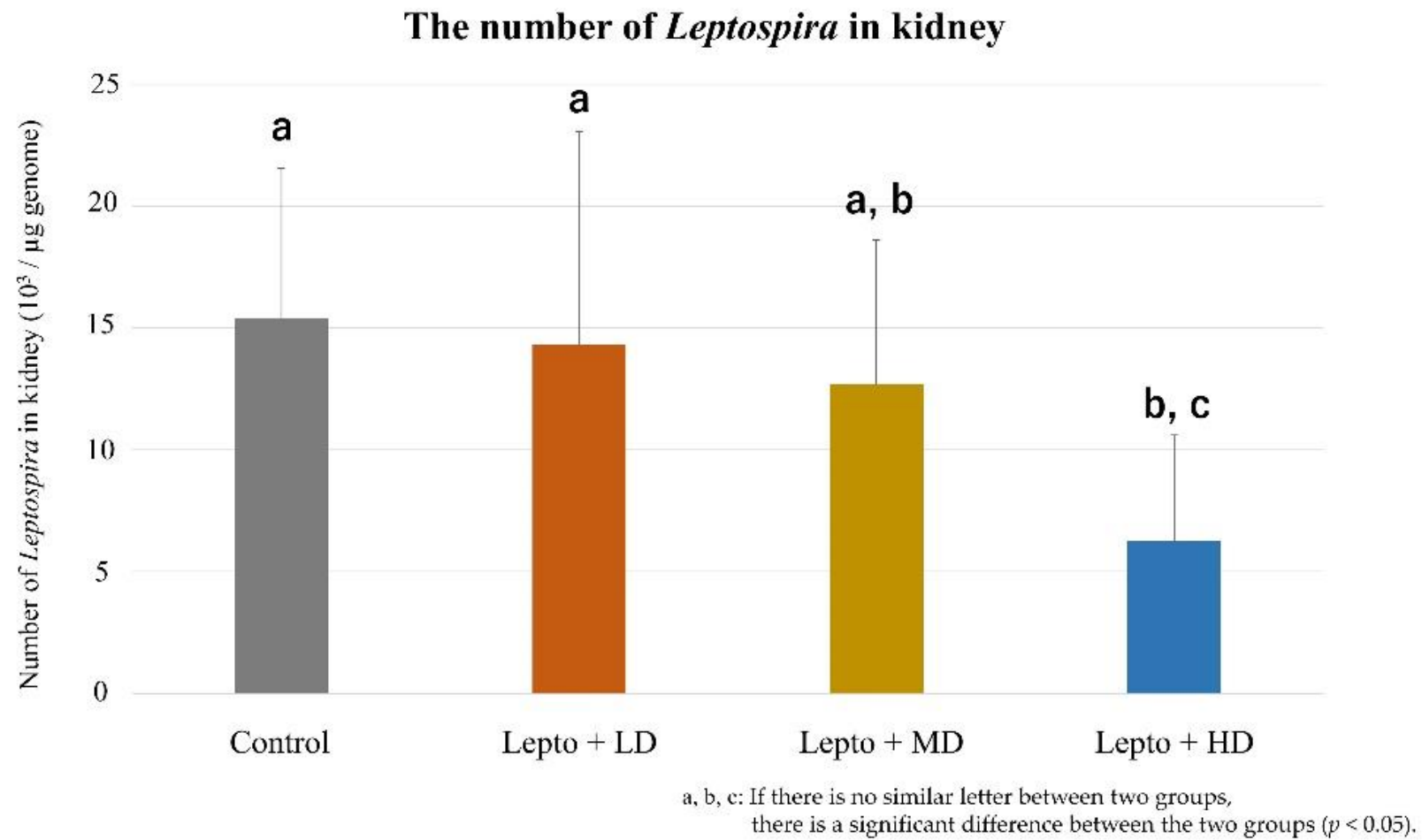


Figure 4-5. Histological examination of kidney tissue.

Histological images of kidneys in each group.

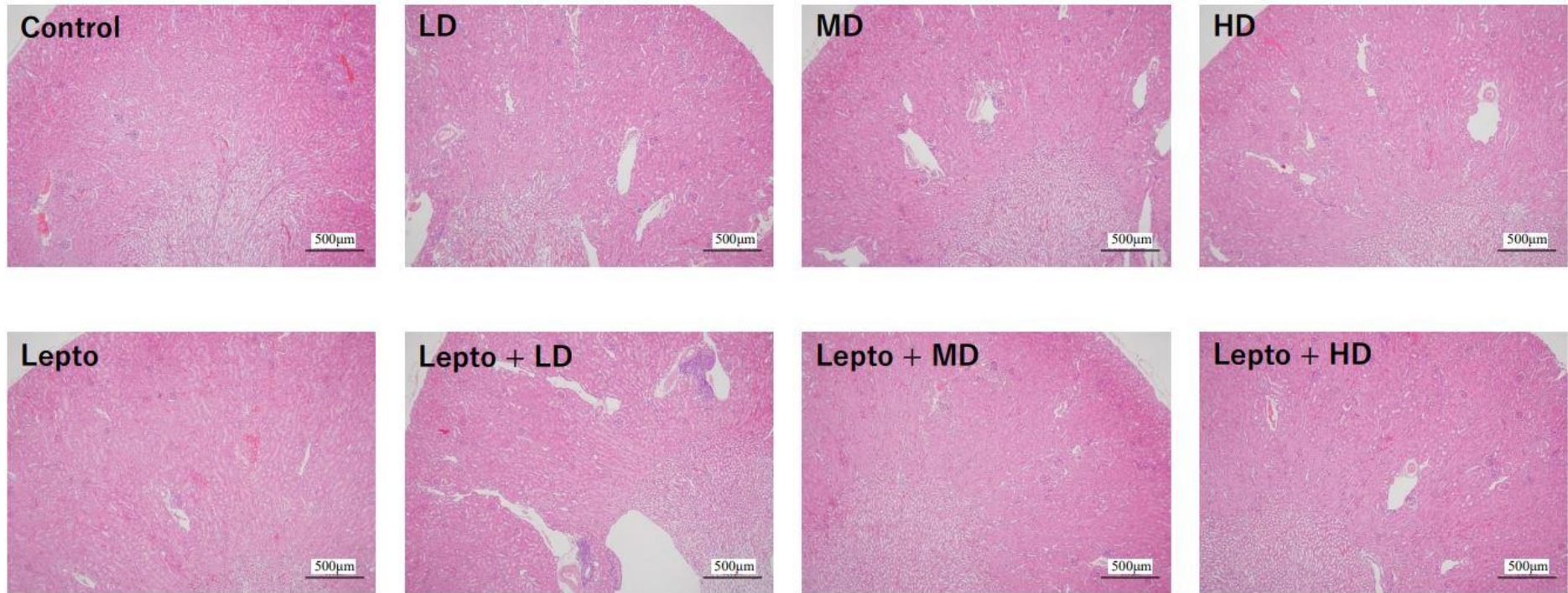
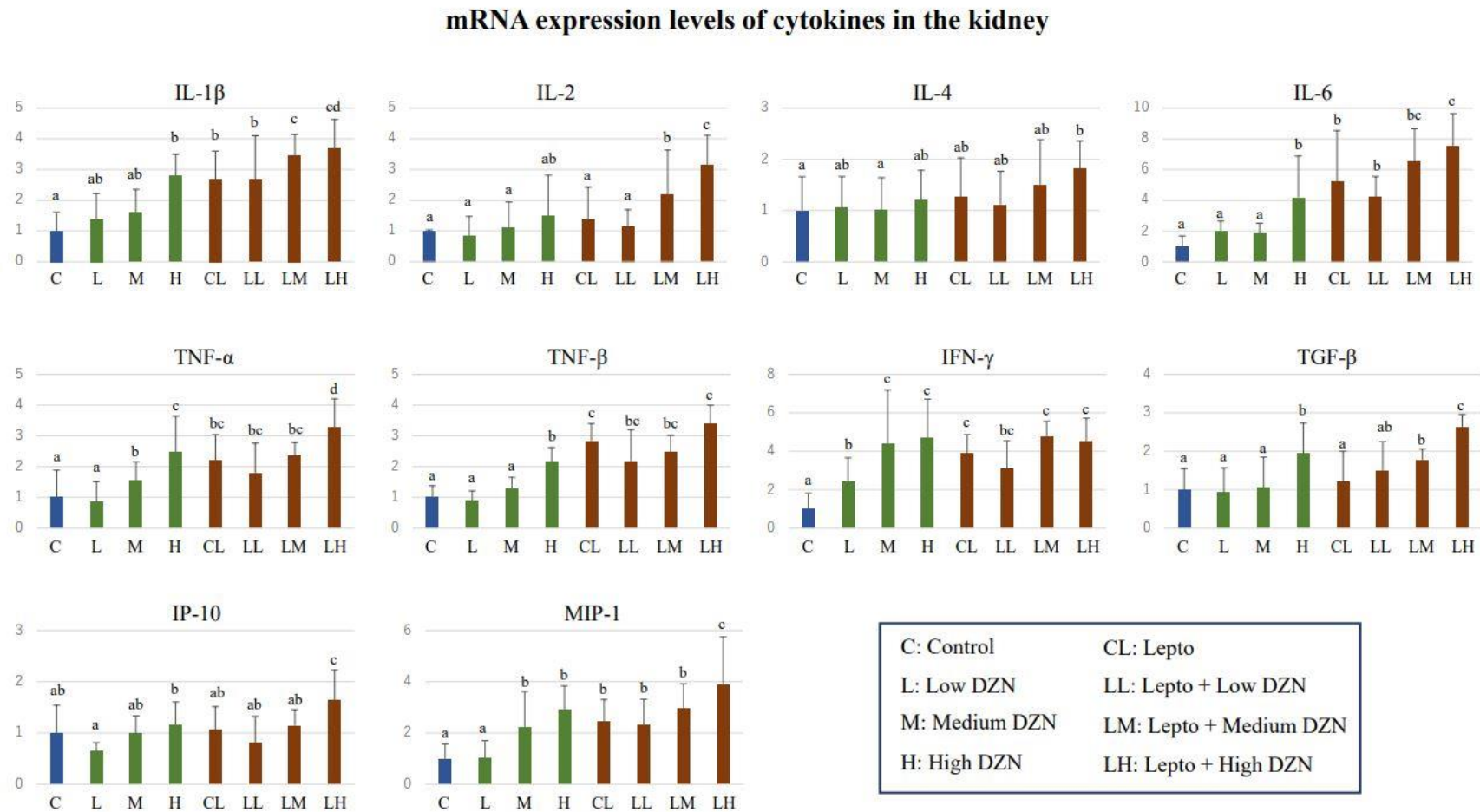


Figure 4-6. mRNA expression levels of cytokine genes in the kidney tissues of diazinon-exposed mice and *Leptospira*-infected mice exposed to diazinon.



Supplementary data

Table S4-1: Sequences of primers used for real-time PCR.

Table S4-2: Multiple-reaction monitoring (MRM) for mass spectrometry.

Table S4-3: Recovery rate and matrix effect of DZN and IM|P in kidney, urine, and EMJH medium ($n = 5$).

Table S4-4: Concentrations of DZN and IMP before and after 5 days of *in vitro* viability assay.

Table S4-1. Sequences of primers used for real-time PCR.

Gene	Primer sequence (5' - 3')		Amplification efficiency (%)
	Forward	Reverse	
RPL18	ATGATGTGCGGATTCTGGAAG	CCTGGGGCCTTGCCAAAAT	96.63
GAPDH	AGGTCGGTGTGAACGGATTTG	GGGGTCGTTGATGGCAACA	95.27
β -actin	GTGACGTTGACATCCGTAAAGA	GCCGGACTCATCGTACTCC	96.39
β -2-microglobulin	TTCTGGTGCTTGTCTCACTGA	CAGTATGTTTCGGCTTCCCATTC	97.28
IL-1 β	GAAATGCCACCTTTTGACAGTG	TGGATGCTCTCATCAGGACAG	101.23
IL-2	TGAGCAGGATGGAGAATTACAGG	GTCCAAGTTCATCTTCTAGGCAC	103.21
IL-4	GGTCTCAACCCCCAGCTAGT	GCCGATGATCTCTCTCAAGTGAT	96.91
IL-6	TCTATACCACTTCACAAGTCGGA	GAATTGCCATTGCACAACTCTTT	96.20
TNF- α	CAGGCGGTGCCTATGTCTC	CGATCACCCCGAAGTTCAGTAG	97.46
TNF- β	TTCTCCGACATGGCTTCTCTT	TGGAAGGGGTATTGGGAGGAA	98.37
IFN- γ	GCCACGGCACAGTCATTGA	TGCTGATGGCCTGATTGTCTT	98.76
TGF- β	CCACCTGCAAGACCATCGAC	CTGGCGAGCCTTAGTTTGGAC	96.03
MIP-1	CTCCCAGCCAGGTGTCATTTT	CTTGGACCCAGGTCTCTTTGG	101.87
IP-10	CCAAGTGCTGCCGTCATTTTC	GGCTCGCAGGGATGATTTCAA	97.63
<i>Leptospira</i> 16S rRNA	CCCGCGTCCGATTAG	TCCATTGTGGCCGAACAC	102.34

Table S4-2. Multiple-reaction monitoring (MRM) for mass spectrometry

Compound	Precursor ion	Product ion	CE	Polarity
DZN	305.01	169.2	24	Positive
		96.8	36	
IMP	153.01	84.0	20	Positive
		43.2	32	

RT: Retention time, CE: Collision energy

**Table S4-3. Recovery rate and matrix effect of DZN and IM|P
in kidney, urine, and EMJH medium (n = 5)**

		Kidney	Urine	EMJH medium
DZN	Recovery rate (%)	84.32 ± 3.21	85.08 ± 5.44	89.66 ± 3.44
	Matrix effect (%)	93.14 ± 2.22	91.31 ± 1.98	94.95 ± 2.26
IMP	Recovery rate (%)	85.21 ± 3.56	96.21 ± 3.32	86.71 ± 4.97
	Matrix effect (%)	99.53 ± 2.54	81.77 ± 1.11	98.65 ± 3.72

Each value is represented as mean ± SD.

Table S4-4. Concentrations of DZN and IMP before and after 5 days of *in vitro* viability assay

	DZN				IMP			
Nominal concentration (ppm)	250	15.6	7.8	1.95	250	15.6	7.8	1.95
Concentration before assay (ppm)	258.9	14.67	6.92	2.03	254.5	14.46	7.11	1.81
Concentration after assay (ppm)	252.6	15.69	6.14	1.98	252.6	15.60	6.59	1.71
Before / After assay ration (%)	102.5	93.5	112.7	102.5	100.8	92.7	107.9	105.8

SUMMARY

The ultimate goal of this research is a contribution to wildlife conservation. The studies presented here focused on risk assessment of pesticide exposure to wild mammals because there are concerns that many wildlife have been affected by pesticides. I elucidated the pesticide contamination situation and the causes of pesticide exposure in raccoons and masked palm civets in Japan, revealed the unique metabolic characteristics of pesticides in musk shrew, and evaluated the effect of pesticide exposure on mice infected with pathogens using mice and *Leptospira*.

In chapter 1, I investigate the presence of NNIs in the urine of wild raccoons in Hokkaido, Japan, and examines their metabolic capabilities compared to rats. The study detected several NNIs and their metabolites in raccoon urine, with CLO being the most abundant. Comparison with human urine samples showed similar levels of NNIs, except for higher concentrations of ACP in raccoons. The study also found a lower metabolic capacity for NNIs in raccoon liver compared to rats, indicating a potentially higher susceptibility in raccoons. Although exposure levels were below established safety thresholds, concerns remain due to potential additive and synergistic toxic effects of multiple NNI exposures. This chapter highlights the need for further research and

biomonitoring to understand the potential impact of NNIs on wildlife populations and ecosystems, taking into account interspecies differences in metabolism and toxicity.

In chapter 2, I investigate the exposure status of various pesticides in wild raccoons in cropland and masked palm civets in metropolitan areas of Japan. The food types and pesticide concentrations in the stomach contents of both species were analyzed, and maximum exposure levels were estimated. Raccoons consumed mainly corn and showed high exposure to neonicotinoid insecticides, while masked palm civets consumed various crops and industrial antifungal agents in addition to neonicotinoids. Although maximum exposure levels remained below ADI of human, potential synergistic effects and acute toxicity risks warrant further investigation. The study highlights the importance of monitoring and managing pesticide use to protect wildlife and ecosystems in altered landscapes, and argues for long-term research into the effects of pesticide exposure.

In chapter 3, I conduct a comprehensive investigation of the genetic characteristics and metabolic responses of musk shrews to the neonicotinoid insecticide ACP. Through phylogenetic and syntenic analyses, the study identifies various CYP genes in musk shrews and elucidates their orthologous relationships with other mammalian species. In addition, the mRNA expression levels of CYP genes in musk

shrew liver are evaluated, revealing high expression levels of certain CYP isoforms.

Pharmacokinetic analyses show different metabolic responses to ACP in musk shrews

compared to mice, with musk shrews exhibiting faster clearance rates of ACP. In

addition, *in vitro* assays demonstrate the involvement of specific CYP isoforms,

particularly CYP2A, in ACP metabolism in musk shrews. These findings underscore the

importance of considering species-specific metabolic traits in the assessment of

environmental contaminants and highlight the potential of musk shrews as a valuable

model for studying pesticide toxicity in wildlife. Overall, the study provides valuable

insights into the unique metabolic responses of musk shrews to ACP exposure and

highlights the importance of incorporating species-specific factors in the toxicological

assessment of pesticides.

In chapter 4, I investigated the effect of pesticide exposure on reservoir animals of pathogens by using *Leptospira* and mice as a model. *In vivo* pesticide exposure study showed a decrease in urinary excretion of *Leptospira* in DZN-exposed mice. Kidney tissue analysis showed lower *Leptospira* levels in the high-dose exposure group compared with controls. DZN exposure resulted in increased mRNA expression levels of several cytokine genes, suggesting an enhanced immune response. The study suggests that DZN exposure may reduce urinary shedding of *Leptospira* in reservoir

animals, potentially reducing the risk of leptospirosis transmission to humans. Further research is needed to investigate the effects of various pesticides on infectious diseases beyond leptospirosis in wildlife populations.

There are many threatened species of wild mammals, and many of them are dying due to the influences of human activity. Pesticide exposure is one of those problems still occurring in the world, including Japan. In EU countries, the government decided to strictly prohibit the use of NNIs, and the increased number of wildlife such as insects and birds which are sensitive to these chemicals have been confirmed.

However, as indicated by the high residue limits in food products, the regulation of pesticide use in Japan is less stringent than in other developed countries. Japan is one of the biggest users of pesticides when comparing pesticide use per land area. Data on pesticide exposure in terrestrial wildlife in Japan are very scarce, and data need to be collected on different species. The appropriateness of current pesticide limits will need to be reconsidered in the light of these data.

In addition, we have to assess interspecies differences of pesticides in various terrestrial wild mammals, especially in carnivore species. Carnivores at the top of the ecological pyramid often have smaller populations and tend to be more sensitive to chemicals than omnivores and herbivores. When assessing the toxicity of pesticides to

wildlife, risk management should be based on data from the most sensitive species.

Research on the detoxification potential of pesticides is limited, but data from top predators need to be aggregated.

Regarding the study of leptospirosis, there are few research which investigate the effects of chemical exposure in infected animals. Reservoir animals are exposed to pesticides in the environment and have not been studied, although they may influence the risk of transmission to humans. Collaborative research between toxicology and microbiology needs to be developed.

This study has shown that pesticide exposure in wild mammals is ongoing in Japan. Although the exposure levels confirmed in this study were not high, there is a possibility that co-exposure to several pesticides could cause toxic effects in wild mammals due to interspecies differences in sensitivity to pesticides. Pesticide exposure could also affect the infection status of some pathogens in reservoir wildlife and alter the infectious risk to humans.

To assess the toxic effects of pesticides on wild mammals, it is necessary to determine both pesticide exposure and sensitivity to pesticides. The difficulty of sampling wild mammals has limited the number of previous studies, but I was able to conduct this study by using exterminated individuals, focusing on model species, and

conducting *in vitro* and *in silico* method. Further studies are needed on the exposure of wild mammals to pesticides in different and diverse areas, on the pesticide metabolic capacity of various mammalian species especially in carnivorous mammals, on the effect of pesticide exposure on reservoir animals of various infectious diseases.

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