Toxicological studies on feline cytochrome P450 associated with environmental chemical exposures

環境化学物質への曝露に関するネコのシトクロム P450 の毒性学的研究

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Lists of abbreviations

4OH-imidacloprid	4-hydroxy-imidacloprid
5OH-imidacloprid	5-hydroxy-imidacloprid
6-CNA	6-chloronicotinic acid
$^{13}C_{12}$	Carbon isotope
β-NADPH	β-Nicotinamide adenine dinucleotide phosphate
ACTB	Beta-actin
AHR	Aryl hydrocarbon receptor
BW	Body weight
cDNA	Complementary deoxyribonucleic acid
Cl	Chlorine
CYPs	Cytochrome P450s
dc-acetamiprid	Descyano-acetamiprid
DL-PCBs	Dioxin-like Polychlorinated Biphenyls
dm-acetamiprid	Desmethyl-acetamiprid
dm-clothianidin	Desmethyl-clothianidin
dn-dh-imdacloprid	Desnitro-dehydro-imidacloprid

dn-imdacloprid	Desnitro-imidacloprid
G6PDH	Glucose-6-phosphate dehydrogenase
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GPC	Gel permeation chromatography
HCl	Hydrochloric acid
IgG	Immunoglobulin G
IUPAC	International Union of Pure and Applied Chemistry
KCl	Potassium Chloride
KOH	Potassium Hydroxide
KPB	Potassium phosphate buffer
MeO-PCBs	Methoxylated Polychlorinated Biphenyls
mRNA	Messenger ribonucleic acid
N-acetyl-dm-acetamiprid	N-acetyl-desmethyl-acetamiprid
nAChRs	Nicotinic Acetylcholine Receptors
NCBI	National Center for Biotechnology Information
OH-PCBs	Hydroxylated Polychlorinated Biphenyls
PCBs	Polychlorinated Biphenyls

PBDEs	Polybrominated Diphenyl Ethers
PXR	Pregnane X receptor
qRT-PCR	Reverse transcription quantitative polymerase chain reaction
SD	Standard deviation
S/N	Signal to noise ratio
TEQ	Toxic equivalent
TH	Thyroid hormones
UGT	Uridine 5'-diphospho-glucuronosyltransferase
XMEs	Xenobiotic-metabolizing enzymes

Preface

Domestic cats are frequently treated with veterinary drugs and are being increasingly exposed to a variety of environmental chemicals. They are known to be particularly sensitive to some drugs and chemical exposures. Knowledge regarding the biotransformation of xenobiotics in cats is required to better elucidate the species sensitivity to, and toxicity caused by, environmental exposures. Cytochrome P450 (CYP) is one of the most dominant metabolic enzymes in phase I of xenobiotic metabolism and can be induced by numerous substances. Consequently, studies on feline CYP isozyme expression related to chemical exposures are necessary to predict the adverse effects forward to drug development and veterinary clinical medication. In this study, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs), and neonicotinoids were chosen as model environmental compounds that cats are exposed to worldwide. The present study aimed to elucidate the mRNA expression of the CYP1–CYP3 families in cat tissues and CYP mRNA expressions related to PCB and PBDE exposures, as well as investigate the differences in CYP activity in the metabolism of PCBs and neonicotinoids between cats and other species.

This Ph.D. thesis includes four chapters:

Chapter 1 presents the general metabolic background of cats, the importance of environmental chemicals they are exposed to, and the role of CYPs in cats. Additionally, the main objective of this study is included at the end of this chapter.

Chapter 2 discusses studies regarding tissue distribution and mRNA expression of feline CYP isoforms associated with *in vivo* organohalogen exposures.

Chapter 3 describes the interspecies differences in specific CYP isoforms responsible for PCB metabolism and investigations on CYP activity for neonicotinoid metabolism in cats compared to other species.

Chapter 4 concludes all outcomes in this study and provides future perspectives based on the results presented in this thesis.

SUMMARY: *In cats, the highest abundance of CYP1–CYP3 (CYP1A2, CYP2A13, CYP2C41, CYP2D6, CYP2E1, CYP2E2, CYP2F2, CYP2F5, CYP2J2, CYP2U1, and CYP3A132) was found in the liver, but some extrahepatic isozymes were found in the kidney (CYP1A1), heart (CYP1B1), lung (CYP2B11 and CYP2S1) and small intestine (CYP3A131). Feline CYP1A1, CYP1A2 and CYP1B1 were significantly upregulated in the liver as well as in several other tissues after a single exposure to PCBs. In contrast, CYP1–CYP3 mRNA expression showed no significant difference between control and BDE-209 exposure groups in cats, which indicated that chronic exposure to BDE-209 did not change CYP expression in the liver of cats. Study of in vitro CYP-mediated PCB metabolism revealed that OH-PCB profiles of cats and dogs were similar and that 4'OH-CB18 was a major metabolite. These findings combined with in silico docking simulation indicated that cat CYP3A and dog CYP3A/1A1 could catalyze most PCBs, particularly PCB18, whereas CYP1A1 in cats and CYP1A2/2B in dogs may contribute less to the metabolism of some PCBs. The levels of OH-PCB formation indicated that feline CYPs have lower affinity for PCBs than canine CYPs. In addition, the kinetic parameters of neonicotinoid metabolism by CYPs indicated that CYP activity for neonicotinoid metabolism is lower in cats than in rats and humans.*

Chapter 1: Introduction

General background

Domestic cats (*Felis catus*) are small carnivorous species belonging to the Felidae family that have become popular companion for human. While the knowledge for veterinary medicine has been developed worldwide, cats are known to be sensitive to acetaminophen treatment and some drugs (Court, 2013b). In accordance with the acetaminophen metabolic pathway (Fig. 1), this drug is mainly detoxified (90%) via glucuronidation and sulfation (Yoon et al., 2016). Therefore, the feline glucuronidation insufficiency involves the UGT1A6 pseudogene and less diversity in UGT1A isoform expression (Court, 2013b; Court and Greenblatt, 2000), which can cause acetaminophen toxicity in cats. In addition, using other non-steroidal anti-inflammatory drugs, which are structurally similar to acetaminophen, in cats is very carefully considered by veterinarians metabolically (Lascelles et al., 2007). Moreover, domestic cats are among the most common pets that share environmental space with humans. Cats can be exposed to various household substances similar to all household members, which may highlight their potential role as a sentinel species for monitoring human exposure to toxic chemicals (Henríquez-Hernández et al., 2017). Recently, biomonitoring

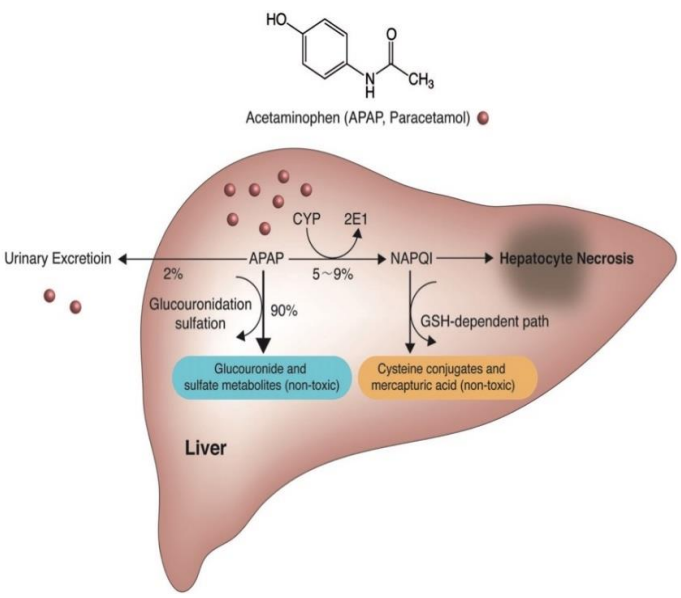

Fig. 1 The metabolic pathway of acetaminophen (Yoon et al., 2016)

studies on household residues and environmental contaminants in cats are of importance and have been reported increasingly (Dye et al., 2007; Henríquez-Hernández et al., 2017; Serpe et al., 2018). Therefore, it is necessary to have extensive studies regarding the exposure and metabolism of cats to xenobiotics to understand the metabolic pathways related to environmental chemical exposure and to protect them from the toxicities of drug and chemical use.

Polychlorinated biphenyls (PCBs) and polybrominated diphenyl ethers (PBDEs) are synthetic organic compounds (Fig. 2) that have been extensively detected in environments (Kodavanti and Loganathan, 2017); they consist of 209 different compounds that depend on the positions of choline or bromine atoms attached to the biphenyl (Siddiqi et al., 2003). According to their chemical properties, PBDEs have been widely used as flame retardants, and PCBs have been applied as coolants and lubricants in transformers and other electrical equipment (Kodavanti, 2017; Kodavanti et al., 2008). Although all PCBs and some PBDE congeners have been banned for use in all electrical and electronic application industries, many toxicities and bioaccumulations have been studied continuously because of their environmental persistence causing numerous opportunities for exposure to humans and their pets. The organohalogens, including PCBs 118, 138, 153, 180, and 187 and PBDEs 47, 99, 153, and 209, are abundant in the sera and hair of domestic cats and dogs (Ali et al., 2013; González-Gómez et al., 2018; Mizukawa et al., 2013; Norrgran et al., 2015; Serpe et al., 2018) as well as in house dust (Braouezec et al., 2016; DellaValle et al., 2013; Guo et al., 2012; Wang et al., 2013; Whitehead et al., 2014). Endocrine system disruptions caused by PCBs and PBDEs have been the primary focus since these chemicals have structures similar

to those of thyroid hormones (THs) and other steroid hormones (Makey et al., 2016; Turyk et al., 2008). PCBs and PBDEs can cause a range of adverse effects on health such as thyroid toxicity, developmental neurotoxicity causing impaired brain development, gonadal developmental toxicity and possibly cancer in human, livestock and wildlife (Costa and Giordano, 2007; Fang et al., 2002; Kaw and Kannan, 2017; Ruder et al., 2017). In cats, many findings suggested that the levels of these organohalogen compounds in the blood are related to feline hyperthyroidism or thyroid hormone disturbance (Mensching et al., 2012; Norrgran et al., 2015; Peterson, 2013). The acute and chronic responses of cats exposed to PCBs and decabromodiphenyl ether (BDE-209) were clarified in our studies (unpublished data). After cats were exposed to 12 PCB mixtures (short-term exposure; single dose; i.p.), I observed a statistical decrease in relative testicular weight and serum levels of albumin and total protein in the exposure group compared to the control group. Negative correlations were found between PCB and hydroxylated metabolite (OH-PCB) levels, and blood thyroid hormone (TH) levels (including total thyroxine and total triiodothyronine). Meanwhile, a significant decrease in serum albumin levels and relative brain weight were found in the long-term BDE-209 exposed cats (one-year oral administration) compared to those in the control cats. In addition, cats exposed to BDE-209 showed an increase in subcutaneous fat and levels of serum high-density lipoprotein (HDL) and triglycerides (TG), and the downregulation of stearoyl-CoA desaturase mRNA expression in the liver. Our findings suggested that chronic BDE-209 exposure may restrain lipolysis in the liver, which is associated with lipogenesis in the subcutaneous tissues. There was no significant difference between the liver enzymes, blood urea nitrogen, and creatinine levels of the two groups in both experiments, which

implies that liver and kidney cell damage was negligible.

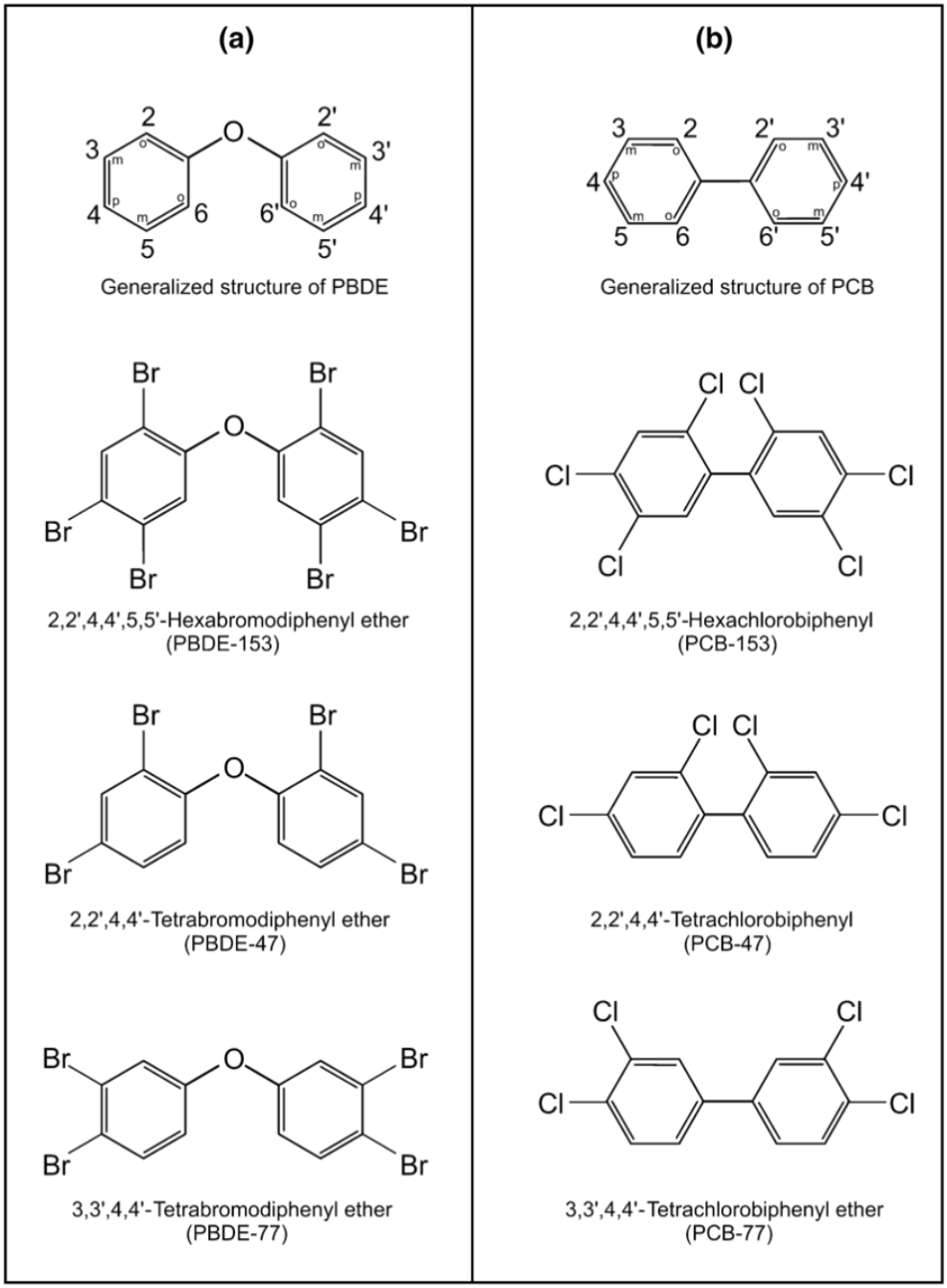

Fig. 2 General structures and some congeners of PCBs (a) and PBDEs (b) (Kodavanti and Loganathan, 2017)

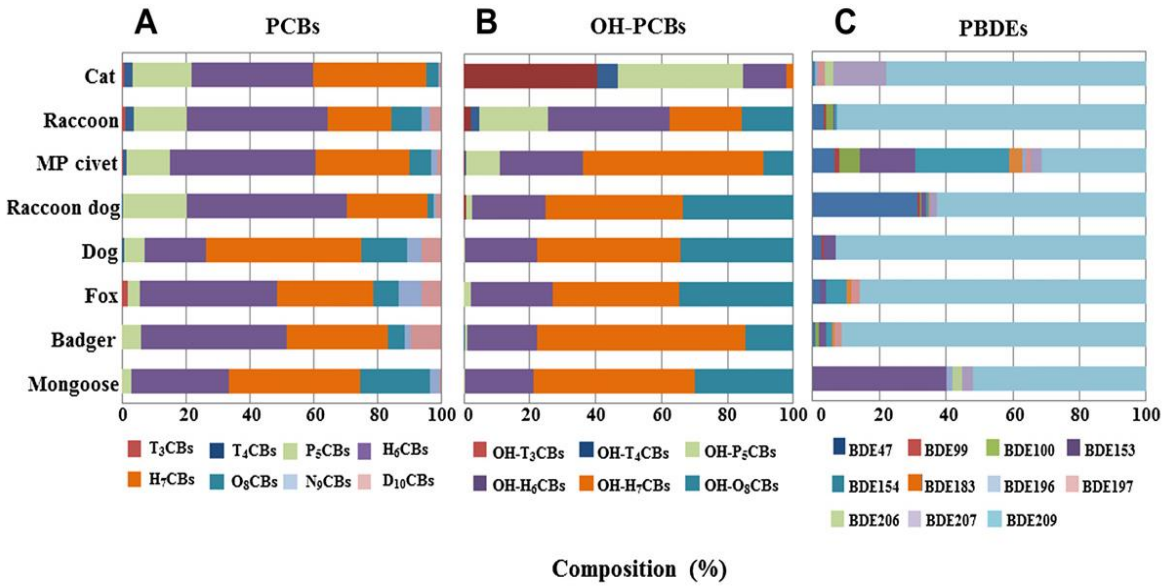

Fig.3 Compositions of PCBs (A), hydroxylated PCBs (B) and PBDEs (C) in the blood of cats compared to other mammal species (Mizukawa et al., 2013)

PCBs in mammals are oxidized into OH-PCBs by the cytochrome P450 (CYP) monooxygenase system (Bhalla et al., 2016; Tehrani and Van Aken, 2014). The OH-PCBs have increased critical environmental concerns because some evidences suggested that they can exert various toxic effects, particularly endocrine disruption, at lower doses than the parent compounds (Kawano et al., 2005; Purkey et al., 2004). In human, the predominant OH-PCB congeners detected in the blood were 4OH-CB187 and 4OH-CB107 followed by 4OH-CB146, 3OH-CB153, and 3′OH-CB138 (6-7 Cl) (Purkey et al., 2004). Interestingly, although the composition of PCBs is quite similar across all species, the pattern profiles of hydroxylated PCBs (OH-PCBs) in the blood of cats differ (Mizukawa et al., 2013); figure 3 showed that cats highly accumulate lower-chlorinated PCBs (3-5 Cl) in the blood, while the blood of dogs and other species accumulate higher chlorinated PCBs (6-7 Cl). These reports

indicated that the differences in PCB metabolism between cats and other species might be associated with CYP expressions and functions. Therefore, determining the interspecies differences of PCB metabolism between cats and other species (e.g., dogs) is greatly required. Furthermore, the feline CYP expression pattern and induction by organohalogen exposure should be clearly understood to determine the risks of PCBs or PBDEs to domestic cats.

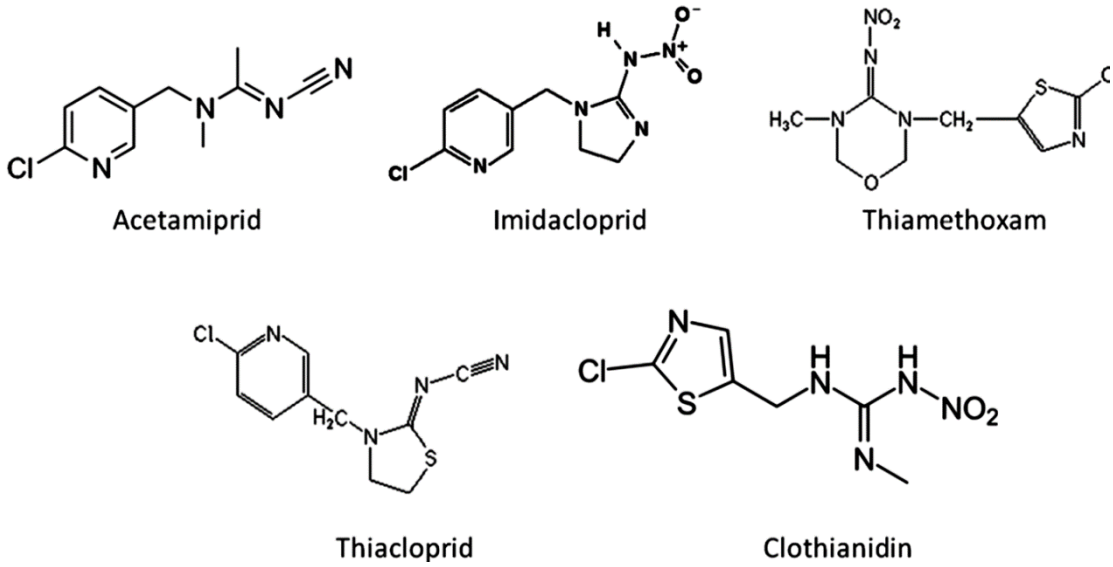

Fig. 4 Chemical structures of some neonicotinoids (Ghanim and Ishaaya, 2011)

In addition to organohalogen chemicals, cats can be directly exposed to veterinary products, including neonicotinoids, which is applied as an effective insecticide to eradicate of ectoparasites, especially cat flea (*Ctenocephalides felis*) (Mehlhorn et al., 2001; Mencke and Jeschke, 2002; Rust, 2005; Vo et al., 2010). Neonicotinoids (Fig. 4) such as imidacloprid, acetamiprid, thiamethoxam, thiacloprid, and clothianidin are a class of commonly used insecticides and are highly water-soluble (Ghanim and Ishaaya, 2011; Morrissey et al., 2015). Neonicotinoids selectively act on the postsynaptic

nicotinic acetylcholine receptors (nAChRs) of insect species, and studies have shown that they have relatively low affinity for vertebrate nicotinic receptors compared to insect nicotinic receptors (Ghanim and Ishaaya, 2011; Thompson et al., 2020; Wood and Goulson, 2017). However, neonicotinoids can be rapidly absorbed through the mammal digestive system causing a variety of toxicities in humans (Simon-Delso et al., 2015; Thompson et al., 2020) and the adverse effects in pets and wildlife, such as frog, rat, rabbit and deer, have been reported as well (Berheim et al., 2019; Gibbons et al., 2015; Sheets et al., 2016). Since neonicotinoids act as neurotoxins, they mainly act on the parasympathetic system and some of the sympathetic system (Selvam and Srinivasan, 2019). In humans, exposure to neonicotinoids can induce various gastrointestinal symptoms (nausea, vomiting, abdominal pain and corrosive lesions), clinical signs associated with the central nervous system (headaches, agitation, confusion, and seizures or coma), cardiovascular signs (tachycardia or bradycardia, hypertension, hypotension and palpitations), and respiratory effects (dyspnea and aspiration pneumonia or respiratory failure) (Lin et al., 2013; Phua et al., 2009; Selvam and Srinivasan, 2019). Neonicotinoids, especially imidacloprid and clothianidin, can cause many adverse effects at sub-lethal doses in wildlife and freshwater vertebrates, ranging from genotoxic and cytotoxic effects, impaired immune function, and reduced growth and reproductive success (Gibbons et al., 2015). However, the toxicity of neonicotinoid exposure in domestic cats and dogs has not been reported in veterinary medicine. A number of studies indicated that the toxicities in mammals might be related to the metabolic capacity of neonicotinoids and accumulation of their metabolites in the brain or other tissues (Casida, 2011; Ford and Casida, 2006; Shi et al., 2009; Thompson et al., 2020). Therefore,

investigating interspecies variations in metabolic capacity (phase I and phase II) to neonicotinoid exposure is vital to understand and estimate the toxicity of metabolites in each exposed species, including pet cats and dogs. To our knowledge, no information is available on the capability for neonicotinoid metabolism in domestic pets, especially domestic cats.

Cytochrome P450s (CYPs) are the biggest superfamily enzymes that are involved in metabolism, such as xenobiotic oxidation and clearance of several compounds in phase I (Otyepka et al., 2011; Zuber et al., 2002). The purpose of the CYP biotransformation process is to convert a substance into inactive metabolites, which are less lipid-soluble, and highly water-soluble so that they are suitable for renal and/or biliary excretion (van Beusekom et al., 2010; Zuber et al., 2002). The CYP structure contains a heme protein as a cofactor that acts as monooxygenases (Fig. 5) (Nair et al., 2016). The CYP superfamily consists of many isozymes, which are classified into gene families based on their amino acid sequences. Among the various CYP families, the CYP1, CYP2, and CYP3 families play an essential role in detoxifying drugs and exogenous chemicals (Tomaszewski et al., 2008; Zanger and Schwab, 2013). The expression and activity of these CYP families for drug uses have been primarily elucidated in rodents, such as mice and rats, as a surrogate for humans in new drug development (Bogaards et al., 2000; Eagling et al., 1998). However, CYP expression and activity differ among age, gender, genetic polymorphism, and animal species (Graham and Lake, 2008; Martignoni et al., 2006; Sadler et al., 2016; Tomaszewski et al., 2008; Zuber et al., 2002). Consequently, investigations on CYP1–CYP3 activities and expression profiles will provide valuable information for predicting environmental chemicals and the effects of pharmaceutical exposure for each species.

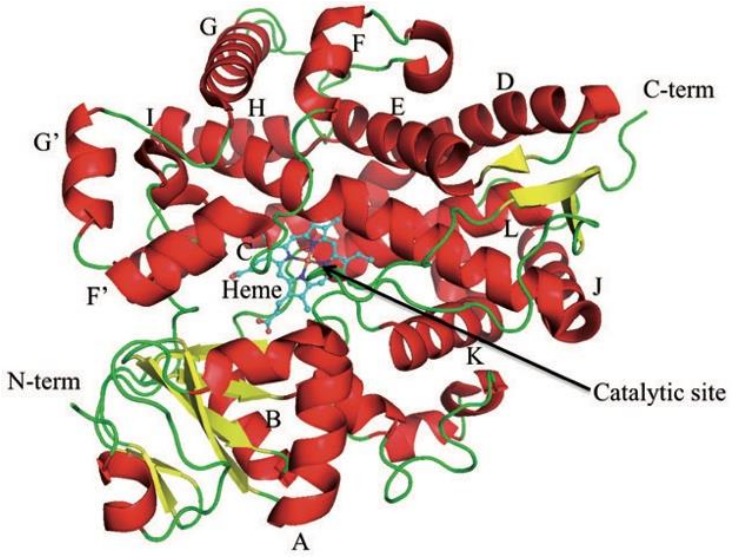

Fig. 5 Structure of cytochrome P450 (human CYP3A4) containing heme (Nair et al., 2016)

In pet cats, studies regarding phase II glucuronidation are very well known; however, knowledge on feline CYP1-3 families, especially toward considerations of specific veterinary drug uses and environmental exposures are relatively limited. Several findings have reported genetic polymorphisms, metabolic activities, as well as protein and mRNA expressions of some feline CYPs, including CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A (Honda et al., 2011; Komatsu et al., 2010; Okamatsu et al., 2017a; Ono et al., 2019; Shah et al., 2007; Sugiyama et al., 2019; Tanaka et al., 2006). In addition, Beusekom et al., (2013) measured the metabolic activities associated with these CYP1-3 families (using fluorescent substrates and classical inhibitors specific for certain isozymes) in the liver microsomes of cats in comparison to dogs and humans and observed that CYP-activities in cats are lower than those in dogs or humans, except for CYP2B. On the other hand, CYP2B expression at the mRNA and protein levels in the liver of cats was not observed in other

studies (Khidkhan et al., 2019; Okamatsu et al., 2017b). Most of these CYP studies had mainly evaluated the liver; however, CYP expression was also found in extrahepatic tissues, which is important for individual cell activity in response to various stimuli (Larsen et al., 1998; Meng et al., 2015; Meyer et al., 2002). Therefore, the study on CYP expression profiles in the hepatic and extrahepatic tissues such as the brain, lung, intestine, and kidney will contribute to knowledge regarding the differences in regulating tissue metabolic activity, susceptibility to xenobiotic exposures, as well as the regional treatment outcomes for domestic cats (Ferguson and Tyndale, 2011; Kamata et al., 2018; Knights et al., 2013).

Objectives

The objectives in this thesis were (1) to elucidate the mRNA expression of the CYP1-3 families in cat tissues that are useful in defining the specific metabolic capacity in each tissue; (2) to investigate the CYP mRNA expression related to *in vivo* organohalogen exposures, including chronic BDE-209 exposure and acute PCB exposure, that can support our knowledge on the toxicity in, and clinical signs of, cats exposed to these chemicals; (3) to estimate the pathways of feline CYP-mediated PCB metabolism to clarify the specific CYP isoforms for PCB in cats compared to dogs; and (4) to study the interspecies differences in CYP metabolic abilities for neonicotinoids between cats and other species that will provide significant evidence to evaluate the capacity of CYP activity and clearance of these chemicals in cats. These objectives will allow us to better understand the chemicals induced CYP, and in the future, progress to toxicity prediction and the ability of CYP metabolism after exposure to several compounds in cats since they are frequently exposed to drugs and environmental pollutants.

Chapter 2: Tissue distribution and mRNA expression of feline cytochrome P450 related to organohalogen exposures

Abstract

Cats have been known to be extremely sensitive to chemical exposures. The knowledge of cytochrome P450 (CYP) expression involved in chemical exposure are necessary in clinical applications for the medication and prediction of adverse effects. However, the characterization of cytochrome P450 (CYP), the dominant enzyme in phase I metabolism, in cats has not extensively been studied. In addition, the information regarding the organohalogens-induced CYP expression in cats is limited. I aimed to elucidate the mRNA expression of the CYP1–CYP3 families in the cat tissues and to investigate the CYP mRNA expression related to PCBs and BDE-209 exposures. In cats, the greatest abundance of CYP1–CYP3 was expressed in the liver, but some extrahepatic isozymes were found in the kidney (CYP1A1), heart (CYP1B1), lung (CYP2B11 and CYP2S1) and small intestine (CYP3A131). In cats, CYP1A1, CYP1A2 and CYP1B1 were significantly upregulated in the liver as well as in several tissues exposed to PCBs, indicating that these CYPs were distinctly induced by PCBs. The strong correlations between 3,3',4,4'-tetrachlorobiphenyl (CB77) and CYP1A1 and CYP1B1 mRNA expressions were noted, demonstrating that CB77 could be a potent CYP1 inducer. All selected CYP isoforms showed no significant difference in mRNA expressions between control and BDE-209 exposure groups, however, CYP3A12 and CYP3A131 revealed tend to be two times higher in the BDE-209 exposure group compared to control group. The present results indicate that the acute exposure of PCBs could clearly upregulate CYP1 family, while chronic exposure of BDE-209 could not alter CYP expression in the liver of cats.

Keywords: Cat, cytochrome P450, mRNA expression, PCBs, BDE-209

Introduction

Cytochrome P450 (CYP) is a large family of heme-containing proteins that play an essential role in the oxidative metabolism of various endogenous compounds, drugs, and xenobiotics. Among several members of the CYP superfamily, the CYP1, CYP2, and CYP3 families are responsible for the biotransformation of most xenobiotics (Ogu and Maxa, 2000; Zanger and Schwab, 2013). Previous studies have revealed that the CYP expression and activity varied among genetic polymorphism, age, and animal species (Graham and Lake, 2008; Martignoni et al., 2006; Sadler et al., 2016; Tomaszewski et al., 2008; Zuber et al., 2002). The strong correlations between mRNA expression levels and enzyme activities of human CYPs (such as CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, and CYP3A4) have been described in the liver (Ohtsuki et al., 2012; Temesvári et al., 2012). As a result, the investigation of CYP1–CYP3 mRNA expression profiles provides valuable information for the prediction of environmental chemicals and pharmaceutical exposure effects for each species.

Domestic cat (*Felis catus*) is one of the most common pets worldwide. This species is highly sensitive to drugs and chemical exposures, probably due to insufficiencies observed in some parts of xenobiotic metabolism (Court, 2013b; Court and Greenblatt, 2000). Glucuronidation deficiency for phase II metabolism is well known in cats, but the study on CYPs, particularly CYP1–CYP3 families, has not extensively been clarified. Several studies have reported the genetic polymorphisms, metabolic activities, and protein and mRNA expressions of some feline CYPs, that is, CYP1A, CYP2A, CYP2B, CYP2C, CYP2D, CYP2E, and CYP3A, have been reported (Honda et al., 2011; Komatsu et al., 2010;

Okamatsu et al., 2017a; Ono et al., 2019; Shah et al., 2007; Sugiyama et al., 2019; Tanaka et al., 2006), and most of these reviewed in the liver. However, the CYP expression is also found in extrahepatic tissues. The CYP expression patterns in the extrahepatic tissues, such as the brain, lung, and kidney, could be determined to understand the variation of controlling the tissue activity, susceptibility to xenobiotics and chemical exposures, as well as the local treatment outcome (Ferguson and Tyndale, 2011; Kamata et al., 2018; Knights et al., 2013). Therefore, the tissue-specific distribution of the CYP expression in cats should be characterized in detail.

CYPs can be induced or inhibited by several drugs and chemicals (Graham and Lake, 2008). Polychlorinated biphenyls (PCBs), which are man-made organohalogen compounds, are known to induce CYPs in humans and animals. Although dioxin-like PCBs (DL-PCBs) have been considered as aryl hydrocarbon receptor (AHR)-related CYP1A inducers (Korytko et al., 1999; Machala et al., 1998; McGraw and Waller, 2006), PCB mixtures are primarily metabolized by CYP2B and CYP3A in fish, rats, and humans (Connor et al., 1995; Koenig et al., 2012; Petersen et al., 2007). Furthermore, following CYP metabolism, the hydroxylated PCB (OH-PCB) metabolites are produced, which are more toxic than their parent compounds (Grimm et al., 2015; Tehrani and Van Aken, 2014). Interspecies differences in the OH-PCB metabolites retained in the blood and tissues have been noted. In numerous terrestrial mammals (e.g., dog, raccoon dog, and fox), the higher-chlorinated hydroxylated metabolites (6–8 Cl-OH-PCBs) were retained in the blood, whereas the lower-chlorinated hydroxylated metabolites (3–4 Cl-OH-PCBs) were mostly detected in the blood of cats (Mizukawa et al., 2013). Moreover, several findings have indicated that the PCBs

exposure contribute to the etiopathogenesis of feline hyperthyroidism (Peterson, 2012; Walter et al., 2017). The causes of feline hyperthyroidism and OH-PCB retention are relatively complicated, and the characterizations of phase I and II enzymes, the distribution and excretion of PCB exposure have been warranted. Considering the specific OH-PCB pattern in the blood and the lack of UGT1A6 and CYP2B in the liver of cats (Kakehi et al., 2015; Okamatsu et al., 2017b), the feline CYP expression pattern and induction by PCB exposure should be clearly understood to determine the risk of PCBs to felines, particularly domestic cats.

Polybrominated diphenyl ethers (PBDEs) are a group of flame-retardants used in numerous household and industrial products (Klincic et al., 2020). They consist of 209 possible congeners that are classified by the number of bromine atoms in the molecule (Schecter et al., 2010; Siddiqi et al., 2003). Due to their structures and chemical properties, PBDEs have persisted in environment, accumulate through food chains, and disturb endocrine systems in animals and humans (Han et al., 2017; Turyk et al., 2008). These compounds have been considered as one of the most harmful organic substances and the production of some congeners (penta-BDE and octa-BDE) has been phased out (Klincic et al., 2020; Schecter et al., 2010), while the deca-BDE (BDE-209) has been generally observed in house dust, egg, poultry meat and human serum (Boucher et al., 2018; Ma et al., 2017; Takigami et al., 2009). However, the toxicity, metabolic process, and CYP expressed in the liver involved in BDE-209 exposure are not clearly understand in several species, even rat and human (Lee et al., 2010; Stapleton et al., 2009; Wang et al., 2012). Cats are one of most popular companions for human and they commonly share the habitats with human being. The

statistical correlations between indoor dust and cat serum levels of chemicals, such as phthalates, BDE-47, BDE-99, BDE-153 and BDE-209, were reported in many studies (Braouezec et al., 2016; Guo et al., 2012; Norrgran et al., 2015). These findings have supported the hypothesis that dust is a significant exposure route for cats. The studies have suggested that pet cats are suitable bio-sentinel for human exposure to household chemicals including PBDEs (Dirtu et al., 2013; Dye et al., 2007; Lau et al., 2017; Mensching et al., 2012). Feline hyperthyroidism associated with increased PBDE levels in cat serum have been reported worldwide (Mensching et al., 2012; Norrgran et al., 2015; Walter et al., 2017), but the information on CYP expression in cat exposed to PBDE has not been provided.

In the present study, I aimed to elucidate the existing isoforms of the CYP1–CYP3 families in various cat tissues including the liver, kidney, heart, lung, small intestine (duodenum, jejunum, and ileum), and brain (cerebrum, cerebellum, hypothalamus, midbrain, pons, and medulla). I also investigated the CYP mRNA expression related to acute PCB exposure in cats using the cDNA cloning and quantitative real-time RT-PCR (qRT-PCR) techniques. To estimate the possible PCB congener-induced CYPs, the correlation between the CYP mRNA expression and DL-PCBs toxic equivalent (TEQ) in the liver was analyzed. Furthermore, I examined the hepatic CYP mRNA expression in cats treated BDE-209 for long-term exposure.

Materials and Methods

Sample collection: PCBs exposure

The animal experiment was carried out in the animal facility that is recognized by the Association for Assessment and Accreditation of Laboratory Animal Care International, and all animal treatments were ethically approved by Hokkaido University, Japan (approval number 14-0054 and 14015). Eight male cats (24–28 months old, *Felis catus*) were purchased from Kitayama Labes Co., Ltd., Japan. They were fed in a 14/10-h light/dark cycle at room temperature (24±5 °C) with a relative humidity of 50±10%. The cats were also fed with water ad libitum and 80 g of food per day. Cat food contained about 3,917 kcal/kg of energy including; 44.5% protein, 9.7% fat, and 2.3% fiber (Nosan Co., Japan). After the 2-month acclimatization, all cats were divided into two groups: control (n = 4, 4.1±0.6 kg) and exposure (n = 4, 4.3±0.3 kg). The cats were intraperitoneally injected with corn oil and a mixture of twelve PCBs (IUPAC no. 18, 28, 70, 77, 99, 101, 118, 138, 153, 180, 187, and 202) in corn oil at a dose of 0.5 mg (each congener)/kg (BW) once in the control and exposure groups, respectively. These twelve PCBs, including DL-PCBs and non-dioxin-like PCBs (NDL-PCBs), have been detected in serum and hair of pet cats and dogs (Ali et al., 2013; González-Gómez et al., 2018; Mizukawa et al., 2013; Serpe et al., 2018); and in house dust (DellaValle et al., 2013; Wang et al., 2013; Whitehead et al., 2014). The exposure dose was adjusted from the PCB dose given to the dogs in a previous study (Korytko et al., 1999). After a 5-day exposure period, the cats were anesthetized with pentobarbital and euthanized by KCl injection. The dissections were performed by a qualified veterinarian. All tissues were collected under aseptic conditions and stored at –80 °C until further analysis. The

contents of the small and large intestines were removed, after which they were washed with phosphate-buffered saline, cut into segments and kept in –80 °C until analysis. The samples used for mRNA expression studies were kept in RNAlater (Sigma-Aldrich Co., St. Louis, MO, USA).

Sample collection: BDE-209 exposure

BDE-209 exposure test was ethically approved by Hokkaido University (approval number 14-0054 and 14015) and were performed using six male cats (13 months old, 3–4 kg, *Felis catus*). The animals were purchased from Kitayama Labes Co., Ltd. (Nagano, Japan) and fed by 80 g of cat foods contained about 3,917 kcal/kg of energy including; 44.5% protein, 9.7% fat, and 2.3% fiber (Nosan Co., Kanagawa, Japan) and unlimited water supply in accordance with the guideline of the Association for Assessment and Accreditation of Laboratory Animal Care International at the Faculty of Veterinary Medicine, Hokkaido University, Japan. The cat foods were analyzed and confirmed that no PBDEs contamination. After three months' acclimatization, they were divided into two groups: control (n = 3) and exposure (n = 3). The exposure group was orally treated by powder BDE-209 in capsule (7 mg/kg bw/week; purity: ≥99%; Wellington Laboratories Inc. Ontario, Canada), while the control group was given by capsule only. This exposure dose was adjusted that referred to environmental exposure levels in the previous studies (McDonald, 2005; Mensching et al., 2012), and dosed every week. After one-year exposure, the cats were anesthetized with pentobarbital and euthanized by KCl injection. The liver sample used for gene expression studies were preserved in RNAlater (Sigma-Aldrich Co., St. Louis, MO, USA).

Cloning and quantitative real-time PCR

The total RNAs of the liver, kidney, heart, lung, intestine (duodenum, jejunum, and ileum), and brain (cerebrum, cerebellum, hypothalamus, midbrain, pons, and medulla) were extracted and cleaned using TRI Reagent® (SIGMA Life Science, USA) and the NucleoSpin® kit (MACHEREY-NAGEL, Germany), respectively. The integrity and quality of mRNA isolates were examined via 1.5% agarose gel electrophoresis and checked using the 28S and 18S ribosomal RNA bands. The amount of RNA was measured using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., USA), and RNA was synthesized for the cDNA cloning-technique using the ReverTra Ace® qPCR RT Master Mix with gDNA Remover (Toyobo Co., Ltd., Life Science Department, Osaka, Japan). The CYP1–CYP3 genes were selected from the National Center for Biotechnology Information (NCBI) databases and plasmids were constructed by using the TOPO TA Cloning kit (Invitrogen, USA). The nucleotide sequences of the PCR product and plasmid were performed by FASMAC Company (FASMAC Co., Ltd., Japan). All CYP1–CYP3 primers, reference gene primers, and primer sequences as well as the PCR efficacy are shown in the Table 1. The qRT-PCR (StepOnePlus Real-Time PCR System, Applied Biosystems, USA) was performed using a 10-μL PCR reaction mixture containing 5 μL of Fast SYBR Green Master Mix (Applied Biosystems, USA), 0.4 μL of 5-μM forward and reverse primers (Thermo Fisher Scientific, Life Technologies Japan Ltd., Japan), 20 ng of cDNA of each tissue, and 2.2 μL of distilled water. The qRT-PCR condition for all target genes was 95 °C for 20 s, followed by 40 cycles of 95 °C for 3 s, and 60 °C for 30 s.

Chemical analysis

Previous reports have described the measurement method for PCBs and OH-PCBs (Eguchi et al., 2014; Nomiyama et al., 2017). Briefly, 2.5 g of four liver samples from PCB-exposed cats were spiked with the $^{13}C_{12}$-labeled internal standards, denatured by 6 M of HCl, and homogenized with 2-propanol and 50% methyl *t*-butyl ether/hexane. After centrifugation, the organic phase was partitioned into neutral and phenolic fractions using 1 M of KOH in 50% ethanol/water. Using gel permeation chromatography (GPC), the lipid in neutral fraction was removed. Thereafter, the GPC fraction containing PCBs was passed through an activated silica gel column. The phenolic fraction was acidified using sulfuric acid and extracted twice using 50% methyl *t*-butyl ether/hexane. Next, the extracted solution containing OH-PCBs was passed through a deactivated silica gel (5% H_2O deactivated) column and then derivatized to methylated compounds (MeO-PCBs) overnight using trimethylsilyl-diazomethane. After removing the lipid using GPC, the derivatized solution was passed through the activated silica gel column, and the MeO-PCBs were eluted with 10% dichloromethane/hexane. A gas chromatograph (6890 series, Agilent Technologies) coupled to a high-resolution (>10,000) mass spectrometer (JMS-800D, JEOL) was used to identify and quantify the target PCBs and metabolites.

Quality assurance for chemical analysis

The target compounds were quantified using the isotope dilution method for the corresponding $^{13}C_{12}$-internal standards (Eguchi et al., 2014; Nomiyama et al., 2017). A procedural blank was analyzed with each batch of 8 samples using the same protocol that

was applied to the samples, to detect any contamination from solvents and glassware. The limit of quantification was defined as the concentration of target compounds that produced a signal to noise ratio (S/N) of 10. Hepatic samples were showed remarkably low recoveries (38±6% for PCBs and 23±4% for OH-PCBs) because of ion suppression due to the matrix contained within liver tissues. However, these samples with high matrix effects were corrected for the influence for foreign substances by $^{13}C_{12}$-labelsd internal standards in this study. MeO-PCBs were quantified when the retention times matched that of the standards within ±0.1 min, the S/N was greater than 10, and the deviation of ion intensity ratio was within 15% of the standards.

Data and statistical analysis

For PCB exposure experiment, sequence alignments were examined using the MEGA7 software. In addition, the gene copy number for each cDNA sample was calculated using standard curves and normalized with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and beta-actin (ACTB) copy numbers. The result of this experiment was presented as the mean ± standard deviation. A statistical analysis was performed using JMP Pro 13 (SAS Institute, USA). The levels of CYP mRNA expressions in the control and exposure groups were tested for normality using the Shapiro-Wilk test and checked for homogeneity of variance using the Levene's test. Wilcoxon test was used to determine the significant differences between the control and exposure groups. Furthermore, the correlations between CYP mRNA expression and DL-PCB TEQ in the liver were analyzed using Spearman's correlation. P value < 0.05 was considered significant.

The quantification of the transcripts from BDE-209 exposure samples was performed by the ΔΔCT method (Livak and Schmittgen, 2001) normalized with GAPDH and ACTB. A statistical analysis was performed by using JMP Pro13 (SAS institute, Cary, NC, USA). CYP mRNA expressions of the control and exposure groups were confirmed for normality using the Shapiro-Wilk test and tested for homogeneity of variance using the Levene's test. Wilcoxon test and student's t-test were used to determine the significant differences between the control and exposure groups. P value < 0.05 was considered statistically significant.

Results: *Tissue expressions of CYPs related to PCBs exposure in cats*

Tissue distribution of CYP isoforms in cats

The mRNA expressions (copy number of target CYP isoform/copy number of reference genes, GAPDH and ACTB) of 17 CYP isoforms in the hepatic and extrahepatic tissues of the control group are given in the Figs. 1 and 2. The expression levels (mean ± SD) of CYP1–CYP3 were as follows: liver, 0.0004±0.0004 to 27.16±16.20; small intestine, 0.000007±0.00001 to 14.90±4.37; lung, 0.00003±0.00004 to 5.84±4.43; kidney, 0.00004±0.00004 to 5.05±1.69; heart, 0.00002±0.00003 to 0.18±0.10; and brain, undetectable to 0.21±0.14. Although most of CYPs were predominantly expressed in the liver, the expressions of some CYP isoforms were greater than those in the liver, including CYP1A1 (5.05±1.69), CYP1B1 (0.18±0.10), CYP2B11 (5.84±4.43) and CYP2S1 (2.24±1.48), and CYP3A131 (14.90±4.37) in the kidney, heart, lung, and jejunum, respectively. On the other hand, all CYP1–CYP3 existed relatively low (copy number of CYP isoform to copy number of reference genes < 1) in the heart and six parts of the brain. In addition, the comparison of CYP isoforms (% of copy number/copy number of reference genes) in the tissues of cats in the control group is shown in Fig. 3.

Liver: The CYP3A family, especially CYP3A132, was most abundant (45.20% [GAPDH normalization] and 54.09% [ACTB normalization]) in the liver, followed by CYP2 (46.32% [GAPDH normalization] and 34.55% [ACTB normalization]) and CYP1 (8.48% [GAPDH normalization] and 11.35% [ACTB normalization]). However, CYP1B1 and CYP2B11 were hardly detected (<0.01%).

Table 1 List of primers for the RT-qPCR analysis

Gene	Accession number	Forward primer	Reverse Primer	Product size (bp)
GAPDH	NM_001009307.1	5’-GGTGATGCTGGTGCTGAGTA-3’	5’-GTCATGAGTCCCTCCACGAT-3’	266
ACTB	XM_023244304.1	5’-ATCAAGGAGAAGCTGTGCTACGT-3’	5’-CGTTGCCGATGGTGATCA-3’	124
CYP1A1	NM_001024859.1	5’-CTCCTGGCCTCCTTTGTCTT-3’	5’-CACCAGATGTGGGTTCTTCC-3’	150
CYP1A2	NM_001048013.1	5’-GCACCTGCCTCTACAGTAGTTGA-3’	5’-GTGGGTTCTTCCCCAAGGTC-3’	200
CYP1B1	XM_023251947.1	5’-TGTTAACCAGTGGTCCGTGA-3’	5’-AGAGCTGCATCTTGGAGAGC-3’	179
CYP2A13	XM_003997781.5	5’-ACCTTCGACTGGCTCTTCAA-3’	5’-GAAGAAGGTGGGATCGATGA-3’	204
CYP2B11	XM_023249708.1	5’-TTCTGCCTCTGTGGGAGAGT-3’	5’-AATTTGAGGTGGAGAGCCAGG-3’	456
CYP2C41	XM_019813568.2	5’-CAGCAGGAAAACGGATTTGT-3’	5’-CCCTTCCTCACAAAGGAACA-3’	195
CYP2D6	NM_001195406.1	5’-AACCTGCTGCAGATGGACTT-3’	5’-GCAGTGTCCTCGCTGTGATA-3’	164
CYP2E1	NM_001048012.1	5’-AGGACCGGTTAGAGATGCCT-3’	5’-CAGGGAACTCCTGGTTGTCA-3’	189
CYP2E2	NM_001048010.1	5’-TCTGCATGGCTATAAGGCGG-3’	5’-CGGAATGCCTCCATCAGGAA-3’	240
CYP2F2	XM_023245944.1	5’-GGTCCACACCACCGAATCTT-3’	5’-ATAAGGAGCCCGTAACGCAG-3’	254
CYP2F5	XM_006941235.4	5’-GCGACTACCCCACCTTTCTC-3’	5’-GGCTTGCCTTCAGTTTTCCG-3’	196
CYP2J2	XM_019839081.2	5’-CCATCTGGACTTTGAGCGGT-3’	5’-GAGTTGTAGGGCGGTTCACA-3’	173
CYP2S1	XM_011289895.3	5’-AACTCCCCTTGATGTGCAAC-3’	5’-GCGAGAGAGAGGGTATGCTG-3’	226
CYP2U1	XM_019829131.2	5’-ACGTGCCCAAGAAGAGAGTG-3’	5’-CACTTTGGTCCCCATTCCGT-3’	198
CYP3A12	XM_019820248.1	5’-TATTCATTCCCCAAGGGACA-3’	5’-TTTGGGTCCAGTTCCAAAAG-3’	164
CYP3A131	NM_001246278	5’-CTTGCCCTTGTCACACTCCT-3’	5’-TAATTGGGACCGGATAGGCTG-3’	392
CYP3A132	NM_001246271	5’-AACTTGCCCTTGTCAGAGTCC-3’	5’-GTCTATGGGAGCCAGATAGGCTG-3’	397

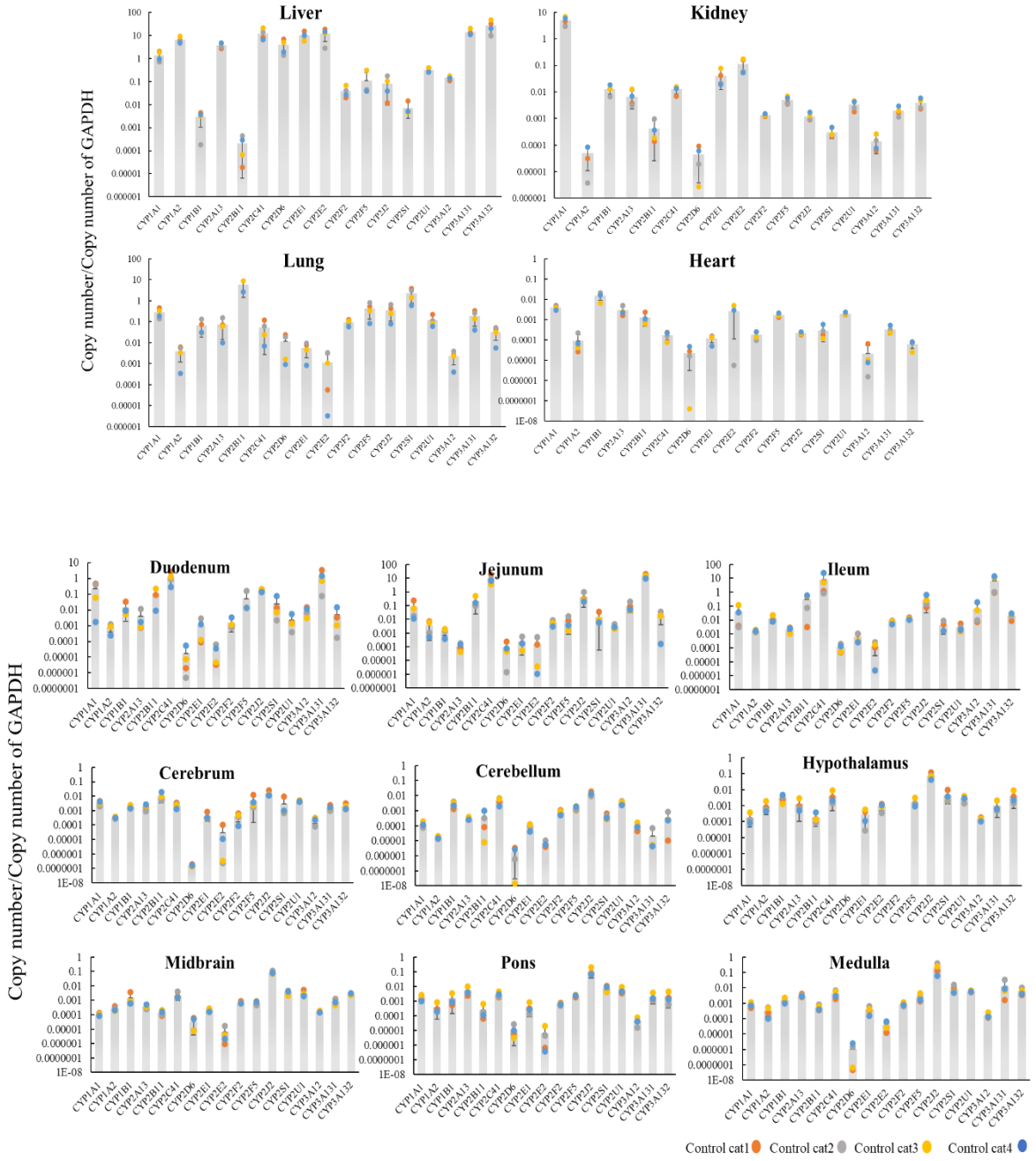

Fig.1 The CYP mRNA expressions (mean ± SD) in the liver, kidney, lung, heart, small intestine, and brain of cats in the control group (Reference genes: GAPDH)

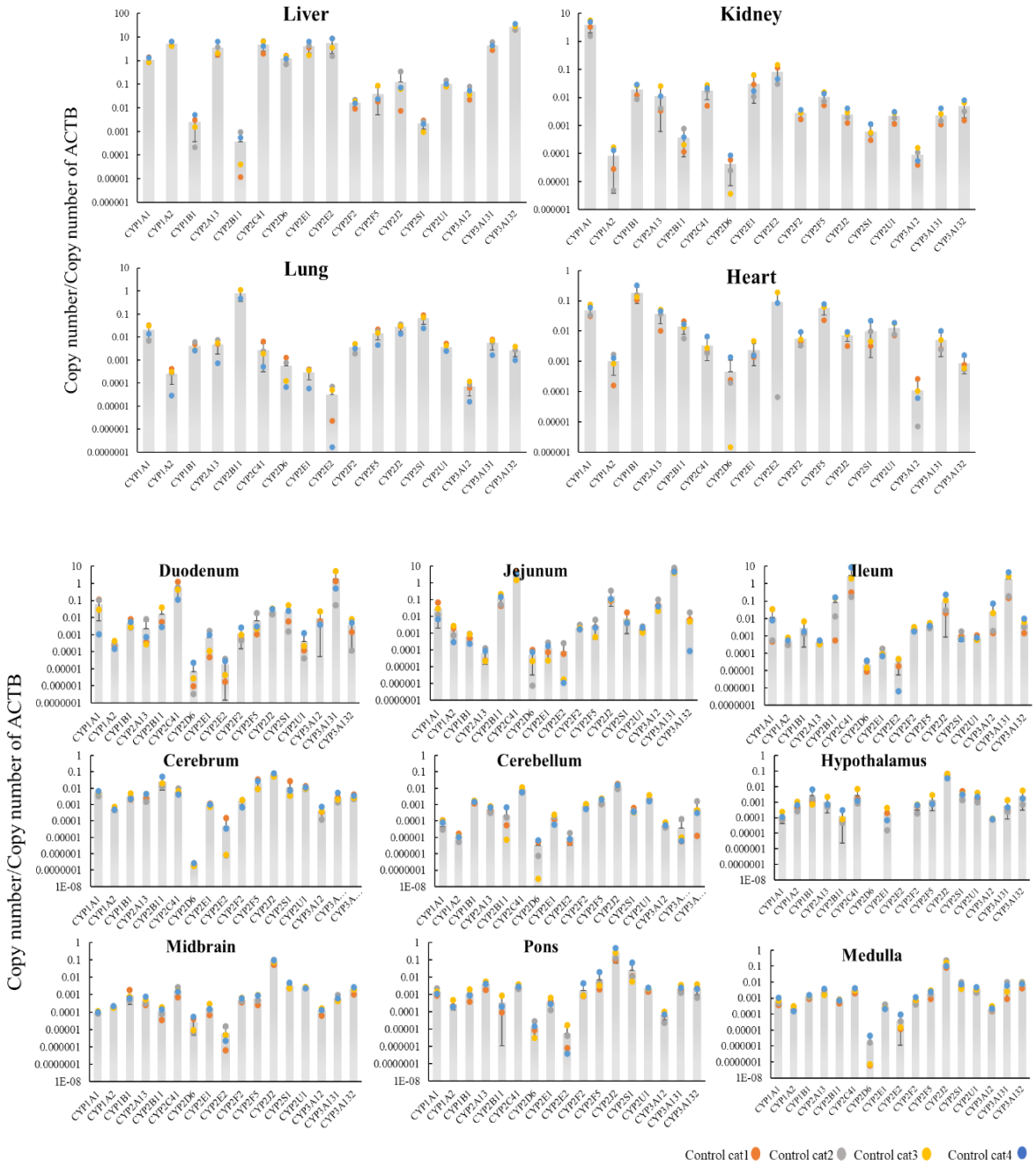

Fig.2 The CYP mRNA expressions (mean ± SD) in the liver, kidney, lung, heart, small intestine, and brain of cats in the control group (Reference genes: ACTB)

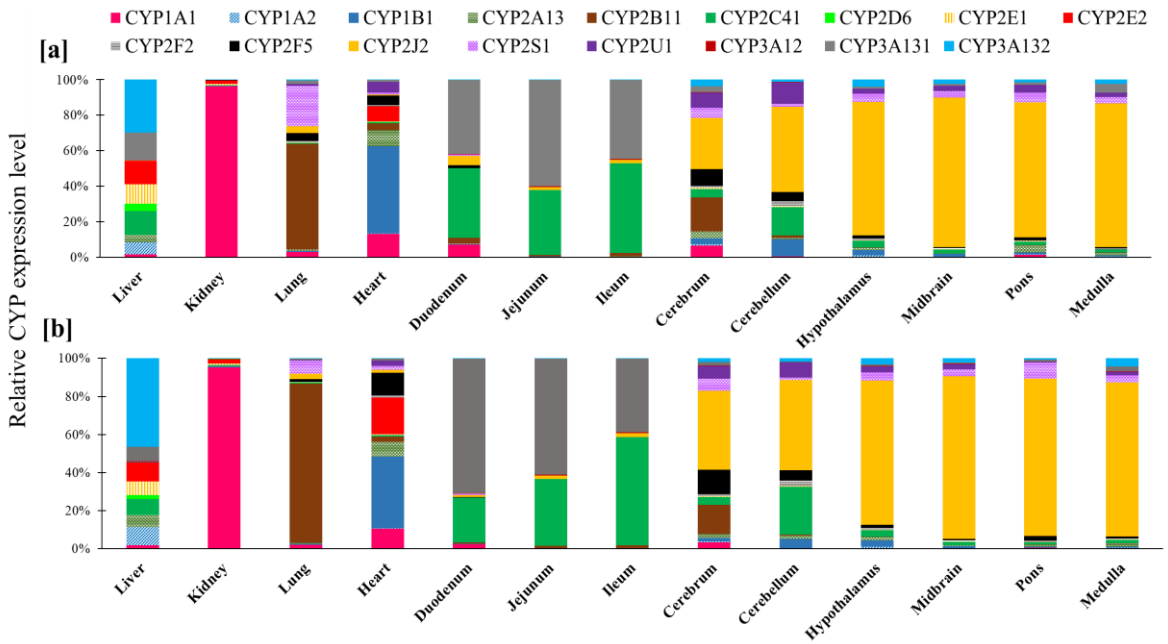

Fig. 3 Comparison of the relative CYP isoforms in the tissues of cats in the control group (Reference genes: GAPDH [a] and ACTB [b])

Kidney: More than 95 % of all CYP expressions in the kidney were CYP1A1 (96.17% [GAPDH normalization] and 95.35% [ACTB normalization]), followed by CYP2 (3.47% [GAPDH normalization] and 4.00% [ACTB normalization]), and CYP3A (0.11% [GAPDH normalization] and 0.18% [ACTB normalization]). Only a scarce number of CYP3A12, CYP1A2, and CYP2D6 (<0.01%), on the contrary, were found.

Lung: CYP2B11 (89.26% [GAPDH normalization] and 94.03% [ACTB normalization]) was highest expressed in the lung, followed by CYP1 (0.95% [GAPDH normalization] and 0.97% [ACTB normalization]) and CYP3A (0.61% [GAPDH normalization] and 0.31% [ACTB normalization]), but only CYP2E2 showed very low expression level (<0.01% [GAPDH and ACTB normalization]).

Heart: In the heart, the dominant CYP isoform was CYP1B1 (49.09% [GAPDH normalization] and 37.79% [ACTB normalization]), and much lower mRNA expressions of CYP2 (36.31% [GAPDH normalization] and 50.26% [ACTB normalization]) and CYP3A (1.31% [GAPDH normalization] and 1.26% [ACTB normalization]) were noted.

Small intestine: In the three parts of the small intestine, nearly 100% of the CYP expressions were CYP3A131 (48.72% [GAPDH normalization] and 56.68% [ACTB normalization]) and CYP2C41 (42.15% [GAPDH normalization] and 38.42% [ACTB normalization]), while the CYP1 expression varied from 0.35% to 7.44 % (GAPDH normalization) and 0.29% to 2.60% (ACTB normalization) in each part. However, the following expressions were sparsely found (<0.01% [GAPDH and ACTB normalization]): duodenum, CYP2E2 and CYP2D6; jejunum, CYP2E1, CYP2E2, CYP2A13, and CYP2D6; and ileum, CYP2E1, CYP2E2, and CYP2D6.

Brain: CYP2J2 (65.55% [GAPDH normalization] and 68.90% [ACTB normalization]) was the main isoform in all six parts of the brain, whereas the expressions of CYP1 and CYP3A were 1.74% to 10.72% (GAPDH normalization), 1.30% to 5.69% (ACTB normalization) and 1.60% to 7.38% (GAPDH normalization), 1.60% to 6.98% (ACTB normalization), respectively. Some CYP isoforms were scarcely observed, including CYP2D6 in the cerebrum and medulla and CYP2E2 in the pons (<0.01% [GAPDH and ACTB normalization]). Moreover, these two isoforms were undetectable in the hypothalamus.

CYP mRNA expressions related to PCB exposure

Comparing the control and exposure groups, the expressions of some CYP isoforms (copy number/reference genes) in each tissue were significantly changed ($P < 0.05$) (Fig. 4 and 5). The significant differences of the CYP1A1 expression between the two groups were found in the liver (5.9- to 6.7-fold), kidney (263.7- to 308.7-fold), lung (9.0- to 9.5-fold), heart (19.7- to 5.3-fold), cerebrum (1.7-fold), cerebellum (50.5- to 57.9-fold), hypothalamus (8.7- to 13.3- fold), midbrain (19.4- to 39.9-fold), Pons (8.6-fold), and medulla (10.1- to 12.2-fold). CYP1A2 showed the significant alteration in liver (2.9- to 3.7- fold), kidney (168.1- to 211.9-fold), heart (6.1-fold), cerebrum (0.6-fold), hypothalamus (45.4-fold), midbrain (2.9-fold), and medulla (2.0-fold) of the PCB-exposed cats compared with those in the control group. The significant CYP1B1 induction was also observed in the liver (37.7-fold), kidney (35.2- to 46.8-fold), lung (25.8- to 37.7-fold), heart (9.7- to 25.6-fold), jejunum (7.7-fold), cerebrum (5.0- to 6.8-fold), cerebellum (5.0-fold), midbrain (3.5- to 7.0-fold), pons (5.6- to 7.8-fold), and medulla (6.5- to 8.0-fold) of PCB-exposed cats compared with those in the control group. Furthermore, the significant differences of the CYP2 expression between the cats in the control and exposure groups were noted as follows: CYP2A13, heart (2.8-fold) and midbrain (2.2-fold); CYP2B11, cerebrum (6.2- to 6.3-fold) and midbrain (1.7- to 2.1-fold); CYP2E2, duodenum (5.0- to 6.1-fold); CYP2F5, ileum (1.4- to 1.5-fold); CYP2J2, midbrain (1.7-fold); CYP2S1, kidney (4.1- to 4.6-fold) and heart (1.8-fold); and CYP2U1, lung (1.7-fold), heart (1.6-fold), medulla (0.7-fold) and pons (0.6-fold). However, CYP3A12 and CYP3A131 in the liver (0.4-fold and 1.58-fold, respectively) and CYP3A131 in the

jejunum (0.3- to 0.7-fold) of cats in the exposure group were significantly downregulated compared with those in the control group.

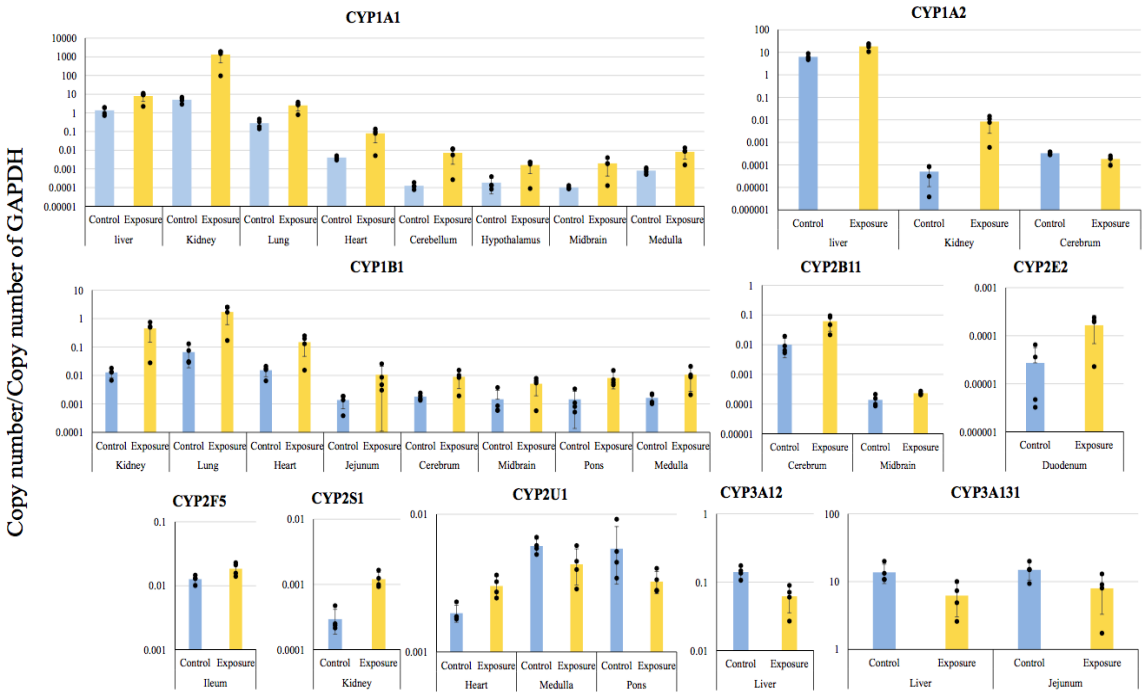

Fig. 4 Comparison of CYP mRNA expressions (mean ± SD) in the tissues of cats between control and exposure groups ($P < 0.05$, Reference gene: GAPDH)

PCB and OH-PCB levels in the liver

Table 2 shows the PCB and OH-PCB levels in the liver samples of cats exposed to PCBs. The mean concentrations (mean ± SD) of the total PCBs and OH-PCBs in the liver were 527.86±462.41 and 209.85±97.46 ng/g, respectively. Among the 12 PCB parent compounds, CB118 (190±270) had the highest level, followed by CB101 (160±230), CB99 (150±230), CB153 (140±210), CB180 (140±200), CB202 (140±190), CB138 (130±190), CB187 (130±190), CB77 (130±160), CB28 (110±100), CB70 (94±150), and CB18 (64±68).

The predominant hydroxylated metabolite in the cat liver was 4'OH-CB18 (130±79), while much lower concentrations of the following were detected: 4′OH-CB61 (28±21), 4OH-CB70 (19±9.9), 4′OH-CB79 (7.6±2.1), 3OH-CB25 (6.1±2.7), 3′OH-CB28 (0.86±1.5), 4OH-CB26 (0.78±0.75), 4OH-CB97 (0.74±0.2), 3OH-CB101 (0.3±0.099), 3OH-CB118 (0.051±0.021), and 4OH-CB187 (0.011±0.019).

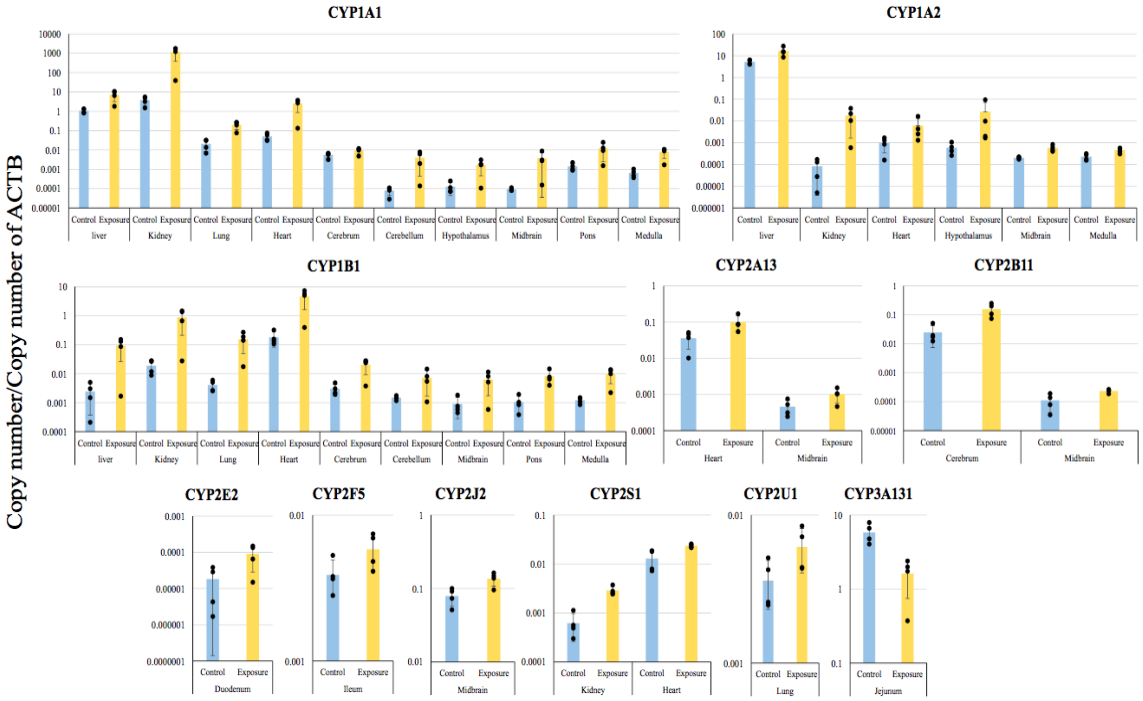

Fig. 5 Comparison of CYP mRNA expressions (mean ± SD) in the tissues of cats between control and exposure groups ($P < 0.05$, Reference gene: ACTB)

Relationship between CYP mRNA expression and DL-PCB TEQ in the liver

Fig. 6 presents the Spearman' correlation coefficients between the CYP mRNA expression levels and TEQ of DL-PCBs (CB77 and CB118) in the liver. Significant correlations were noted between the CYP1A1 mRNA expression level and TEQ of CB77 (ρ

= 1.00) and total TEQ (ρ = 1.00) ($P < 0.05$). Furthermore, the CYP1B1 mRNA expression level was significantly correlated with the TEQ of CB77 (ρ = 1.00) and total TEQ (ρ = 1.00) ($P < 0.05$).

Table 2. Mean concentration of PCBs and OH-PCBs (ng/g wet weight, mean ± SD) in the liver of PCB-exposed cats (n=4).

PCB congeners	**Level** (ng/g wet weight)
CB18	64±68
CB28	110±100
CB70	94±150
CB77	130±160
CB99	150±230
CB101	160±230
CB118	190±270
CB138	130±190
CB153	140±210
CB180	140±200
CB187	130±190
CB202	140±190
Total PCBs	1270±2020
OH-PCB congeners	**Level** (ng/g wet weight)
4'OH-CB18	130±79
3OH-CB25	6.1±2.7
4OH-CB26	0.78±0.75
3'OH-CB28	0.86±1.5
4'OH-CB25/4'OH-CB26/4OH-CB31*	1.5±1.6
4'OH-CB61	28±21
4OH-CB70	19±9.9
4'OH-CB79	7.6±2.1
4OH-CB97	0.74±0.2
3OH-CB101	0.3±0.099
4OH-CB107/4OH-CB108*	0.72±0.02
3OH-CB118	0.051±0.021
4OH-CB101/4OH-CB120*	0.77±0.31
4OH-CB187	0.011±0.019
Total OH-PCBs	200±120

*These OH-PCB congeners could not separate completely by the retention time

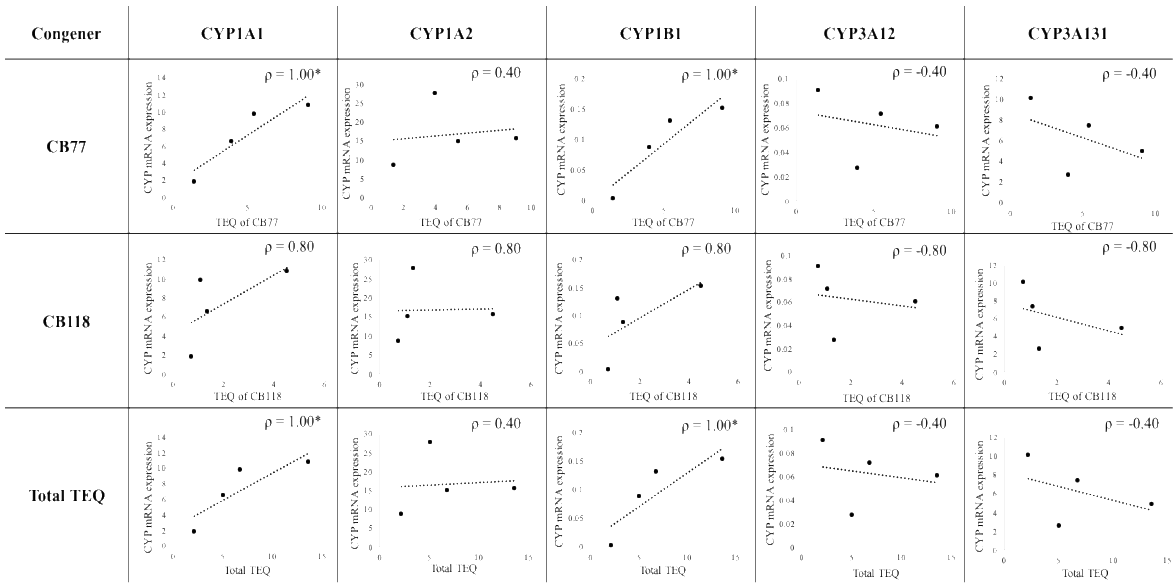

Fig. 6 The Spearman's correlation coefficients (ρ) between the DL-PCB TEQ and the relative CYP mRNA expression levels in the liver of PCB-exposed cats ($P < 0.05$)

Discussion: *Tissue expressions of CYPs related to PCBs exposure in cats*

Tissue expressions of CYP1-3 families in cats

In this study, I focused on the CYP1–CYP3 families because they are the most significant CYPs, which play an essential role in the xenobiotic metabolism in humans and animals (Zanger and Schwab, 2013). In fact, the data on the characterization of the feline CYP1–CYP3 families, including CYP1A1, CYP1A2, CYP2A, CYP2B11, CYP2D6, CYP2E, CYP3A131, and CYP3A132, in the liver have been published (Honda et al., 2011; Komatsu et al., 2010; Okamatsu et al., 2017a; Okamatsu et al., 2017b; Tanaka et al., 2006; Tanaka et al., 2005), but no information on the tissue distribution of all CYP1–CYP3 has been presented. Therefore, after searching feline CYP databases from NCBI website and

primer screening using the conventional PCR, 17 CYP isoforms were selected in this study including CYP1A1, CYP1A2, CYP1B1, CYP2A13, CYP2B11, CYP2C41, CYP2D6, CYP2E1, CYP2E2, CYP2F2, CYP2F5, CYP2J2, CYP2S1, CYP2U1, CYP3A12, CYP3A131, and CYP3A132. GAPDH and ACTB were used as the house keeping genes. Because the stability of reference genes differs among feline tissues (Kessler et al., 2009; Penning et al., 2007), the variations of the relative CYP expressions in the tissues were found in this study.

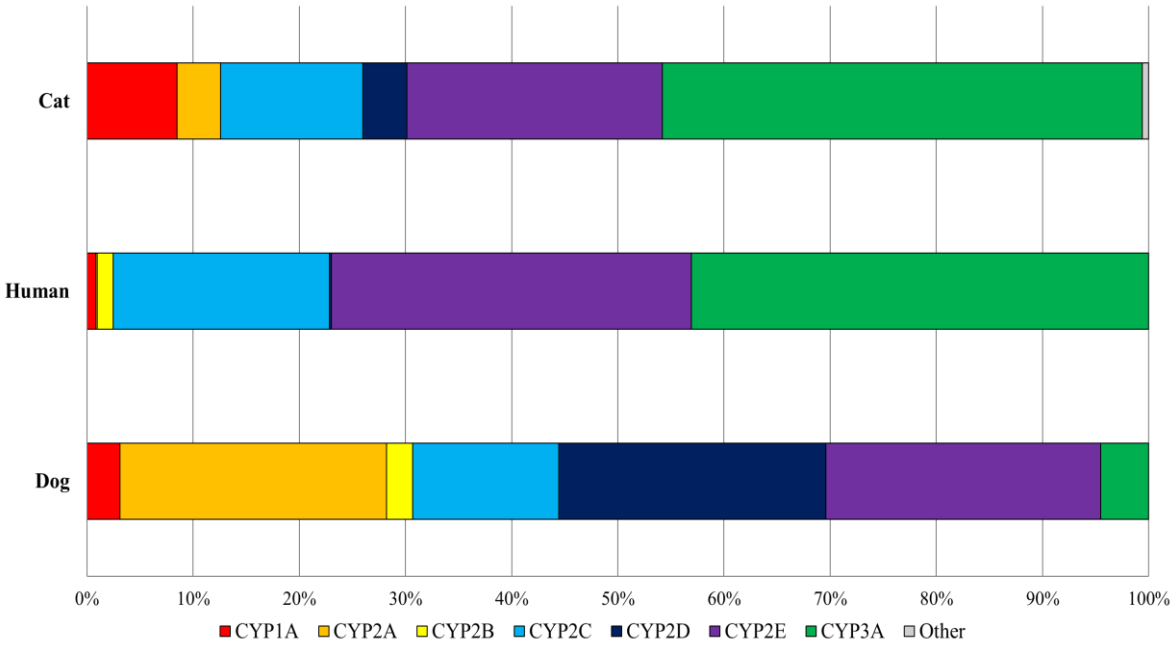

Fig. 7 Comparison of the relative mRNA expressions of the CYP1–CYP3 families in liver of cats (copy number/copy number of GAPDH), humans (copy number/copy number of ACTB) (Rodriguez-Antona et al., 2001), and dogs (copy number/copy number of GAPDH) (Martinez et al., 2013)

CYP expressions in the liver: In the present study, CYP isoforms were mainly expressed in the liver, and CYP3A132 was the most abundant isoform. On the other hand, CYP3A131 and CYP2E2 have been reported as the most dominant isoforms by early studies (Honda et al., 2011; Okamatsu et al., 2017a). The differences in the breed, age, and individual conditions could be the significant factors affecting CYP expressions (Zanger and Schwab, 2013). Moreover, the species difference affects the mRNA expression pattern of CYPs. The comparison of the mRNA expressions of the CYP1–CYP3 families in the liver of humans (Rodriguez-Antona et al., 2001), dogs (Martinez et al., 2013), and cats (present study) is shown in Fig. 7. The mRNA expression of CYP2, including CYP2A, CYP2D, and CYP2E, was dominant in the dog liver, whereas the major isoform in the cat and human livers was CYP3A. In humans, low CYP2A and CYP2D levels were expressed (0.13% and 0.18%, respectively), but the presence of these CYPs in dogs (25.14% and 25.17%) and cats (4.13% and 4.16%) suggested these CYPs are significant subfamily for the xenobiotic metabolism in dogs and cat compared with humans. On the other hand, a lack of CYP2B in the cat liver was noted, and this could alter the metabolism process for some treatment drugs and environmental pollutants such as cyclophosphamide, antipyrine, DDT, and organohalogen compounds (Hedrich et al., 2016).

CYP expressions in the kidney: Some CYP isoforms were mainly expressed in the extrahepatic tissues. The abundance of CYP1A1 in the feline kidney, rat, and mouse kidneys (El-Sherbeni and El-Kadi, 2014; Meyer et al., 2002) suggested that this isoform plays an essential role in the xenobiotic metabolism in their kidneys (Androutsopoulos et al., 2009). CYP1A1 is also primarily expressed in the human kidney. It is used as an indicator for human

renal cell tumor because it metabolizes carcinogens to their inactive derivatives and infrequently converts the chemicals to more potent carcinogens (Cheung et al., 1999; Meng et al., 2015). Therefore, the high-level of mRNA expression of feline CYP1A1 in the kidney may result in an active metabolite-induced renal toxicity. In rats, CYP activities, such as aromatic oxidation and oxidative dehalogenation, in the kidney were equal to or less than those in the liver (Lohr et al., 1998). At the same time, the previous study characterized the feline CYP1A1 functions and found that feline CYP1A1 contributed to theophylline 3-demethylation with high V_{max} and low K_m, in contrast to that of rats (Tanaka et al., 2006). Thus, the species differences of activities in CYP1A1 isolated in the kidneys should be taken into consideration, and further study of these on cats is needed.

CYP expressions in the lung: CYP2B11 expression was found to be predominantly in the lung. It has been suggested that CYP2B11 to play a role in the local defense mechanism of cat's respiratory system (Okamatsu et al., 2017b). In humans, CYP2B6 (the human ortholog of feline CYP2B11) has been primarily expressed in the liver (Rendic, 2002), while CYP2B11 was significantly expressed in both the liver and intestine of dogs (Court, 2013a). Canine CYP2B11 is mainly responsible for the clearance of anesthetic drugs, especially propofol (Hay Kraus et al., 2000). Hence, feline CYP2B11 may not play a central role in the systemic metabolism and clearance of xenobiotics. In addition, a higher CYP2S1 level was found in the lung tissue compared with the liver. In the humans, CYP2S1 was highly expressed in the extrahepatic tissues that were exposed to the environment (e.g., skin, respiratory, urinary, and gastrointestinal tracts) and selectively played a role in pulmonary xenobiotic biotransformation (Bui and Hankinson, 2009; Hukkanen et al., 2002). Human

CYP2S1 was suggested to have a potential role in the carcinogen metabolism and shown to be inducible by 2,3,7,8-tetrachlorodibenzo-p-dioxin through the AHR signaling pathway (Bui and Hankinson, 2009). Therefore, the CYP2S1 expression in the cat lung of cat may have a high metabolic rate for inhaled substances causing cancer.

CYP expressions in the small intestine: CYP3A131 and CYP2C41 were abundantly expressed in all parts of the small intestine. Previous reports (Honda et al., 2011; Okamatsu et al., 2017a) found that the feline CYP3A131 expression in the small intestine was comparable with that in the liver, suggesting the importance of CYP3A131 in xenobiotics that were orally exposed in cats. However, the present study indicated that not only CYP3A131 but also CYP2C41 might play a major role in both hepatic and intestinal xenobiotic clearances. In humans, the high levels of mRNA and protein expressions of CYP2C and CYP3A4 were detected in the small intestine, and the contents of both CYPs decreased toward the small intestine's distal end (Zhang et al., 1999). In contrast, there was greater expression of feline CYP3A131 and CYP2C41 in the jejunum and ileum and then lower expressed upward the duodenum. The distributions of most CYPs varied as a function of position along the small intestine; however, the CYP expression pattern among these three parts of the small intestine is not significantly different in our study.

CYP expressions in the heart and brain: Comparing all tissues analyzed, CYP isoforms were hardly observed in the heart and six parts of the brain. In humans, the total CYP level in the brain was also low (approximately 0.5%–2% of that in the liver); thus, the CYP-mediated metabolism in the brain unlikely affects the systemic metabolite levels

(Ferguson and Tyndale, 2011; Hedlund et al., 2001). Additionally, the metabolic capacity and xenobiotic clearance are relatively low in the mammalian heart compared with those in the liver (Chaudhary et al., 2009). These previous reports confirmed our results that the heart and brain may not play a role in systemic xenobiotic metabolism in cats.

In this study, the dominant CYP isoform in the cats' heart was CYP1B1. Similar to the hearts of humans, rats and mice, the CYP1B1 mRNA expression was also observed in relatively high amounts and associated with the biosynthesis of various intermediated types of arachidonic acid (Chaudhary et al., 2009; Elbekai and El-Kadi, 2006). In addition, the cardiac expressions of CYP1A, CYP2B, CYP2C, CYP2D, and CYP2J are involved in the xenobiotic metabolism within the heart itself (Chaudhary et al., 2009; Thum and Borlak, 2000). Consequently, the CYP mRNA expression pattern in the cat heart indicated that the xenobiotic metabolism could occur in the heart, potentially influencing toxicological efficacy or adverse drug effects.

Although the total CYP levels in the brain are lower than those in the liver, the brain CYPs play an essential role in the local xenobiotic biotransformation, modulating brain activities in specific regions and cell types (Ferguson and Tyndale, 2011; Ghosh et al., 2016; Hedlund et al., 2001). In our results, CYP2J2 was the main isoform in all parts of the cats' brain. Furthermore, in the human brain, CYP2J2 was mainly expressed in the endothelial cells of the cortex and microvessels, while rat neuron cells in the cerebellum, hippocampus, cerebral cortex, and brain stem were the main regions of the CYP2J9 expression (Ghosh et al., 2016). CYP2J is responsible for the metabolism of endogenous fatty acids, whereas the CYP1A, CYP2C, and CYP2B subfamilies contribute to the metabolism of both endogenous

and exogenous substances such as steroids, neurotransmitters, antidepressant drugs, and neurotoxins in the brain (Ferguson and Tyndale, 2011). Therefore, these CYP expression patterns in the cat brain could potentially have a significant impact differently on the local xenobiotic metabolism in each part.

The expressions of CYP related to PCB exposure in the liver

To date, PCB-induced CYP expressions in cats have not yet been reported. The PCB exposure with the experimental dose statistically induced CYP1A1, CYP1A2, and CYP1B1 expressions in both hepatic and extrahepatic tissues (including the kidney, heart, and lung and some parts of the brain), suggesting these CYP isozymes play a fundamental role in the PCBs metabolism in cats. The CYP1 family is generally induced by polycyclic and halogenated aromatic hydrocarbons including dioxin and DL-PCBs involved in the AHR-mediated signaling pathway. The AHR functions contribute to a broad range of physiological roles, including immune function, organ development, reproduction, and steroid signaling modulation (Beischlag et al., 2008). Subsequently, the CYP1A1, CYP1A2, and CYP1B1 expressions should be considered a biomarker not only for the exposure of DL-PCBs (CB77 and CB118) but also for the related toxic effects of these 12 PCB congeners in cats. Furthermore, CYP2B and CYP3A most probably play a major role in the metabolism of NDL-PCBs via the constitutive androstane receptor and pregnane X receptor (PXR) in humans and other animals (Ariyoshi et al., 1995; Gahrs et al., 2013; Koenig et al., 2012; Korytko et al., 1999; Petersen et al., 2007). The inductions of ethoxyresorufin-O-deethylase (CYP1A1) and pentoxyresorufin-O-dealkylase (CYP2B) activities as well as the levels of

NDL-PCB (0–20.5 ng/g) were reported in the livers of two purpose-bred mongrel dogs (age, 12–15 months; weight, 12–27 kg) after PCB exposure (25 mg/kg) (Korytko et al., 1999). The NDL-PCB levels (>0.0074-160 ng/g) in the livers of PCB-exposed cats (24 mg/kg) were relatively higher than those of PCB-exposed dogs, but findings from our study and a previous study (Okamatsu et al., 2017b) suggest that, feline CYP2B does not exist in the liver. These results suggest that NDL-PCBs might be metabolized by only hepatic CYP1A1 in cats and dogs. However, it is possible that the absence of CYP2B expression in cats might be responsible for the reduced biotransformation efficiency of NDL-PCB biotransformation in the cat liver.

The downregulation of CYP3 related to PCB exposure: Relative to the cats in the control group, CYP3A12 and CYP3A131 had statistically lower mRNA expression levels in the liver of PCB-exposed cats, suggesting that PCBs induce a repressor, which is related to the PXR-mediated repression of CYP3A. Although CYP3A downregulation by PCBs in both *in vivo* and *in vitro* studies has not been reported, the hepatic CYP3A expression was suppressed by the parathyroid hormone in rats and during the inflammation by interleukin 6 in the human cell line (Jover et al., 2002; Watanabe et al., 2017). Multiple signaling pathways such as phosphatidylinositol 3-kinase, protein kinases C and A, and nuclear factor kappa B have been reported to be involved in the PXR inactivation, leading to the CYP3A repression (Ding and Staudinger, 2005; Jover et al., 2002; Watanabe et al., 2017). In humans, cattle, and fish, the expressions of CYP3A protein and activity were induced by PCBs (Koenig et al., 2012; Machala et al., 1998; Petersen et al., 2007; Pondugula et al., 2009). Thus, cats could have a specific PCB metabolism as well as toxic effects compared with other animals that

may be consistent with the finding of a specific pattern of OH-PCB metabolites in the cat blood (Mizukawa et al., 2013).

The upregulation of CYP1 related to PCB exposure in the kidney

Xenobiotic biotransformation by CYP occurs not only in the liver but also in the kidney (Lohr et al., 1998). In our results, the kidneys of PCB-exposed cats presented significantly higher mRNA expression levels of CYP1A1, CYP1A2, and CYP1B1 than those in the control group, and the AHR signaling pathway may have contributed to this induction. In the previous reports of mice and rats (Anders, 1980; Lohr et al., 1998), PCBs (Aroclor 1254) slightly induced the renal oxidase activity (2.1-fold), whereas dioxin (TCDD) produced a drastic oxidase activity induction (55.6–60.4-fold), suggesting that DL-PCBs have a higher potential to induce the renal CYP1 mRNA expressions compared with NDL-PCBs. In addition, CYP1A1 in the human kidney is one of the most active CYPs in metabolizing procarcinogens to highly reactive intermediates that can cause cancer and other toxicities (Meng et al., 2015). Therefore, the toxicological consequences of the CYP mRNA inductions in the kidneys of PCB-exposed cats should be considered as well.

The expressions of CYP2 family related to PCB exposure in extrahepatic tissues

Unlike other tissues, the intestinal CYP1A mRNA expressions were not altered by PCBs, suggesting that, gut flora might possibly be causing the specific expression of CYP in the intestines of PCB-exposed cats. The metabolic capacity of enteric bacteria has been reported to alter the expression and activity of CYP in the liver as well as in the small intestine (Bezirtzoglou, 2012; Björkholm et al., 2009; Ishii et al., 2012). However, the upregulation of

the CYP2 family were observed in the intestines and other tissues of PCB-exposed cats. In humans, the CYP2 family is significantly responsible for the CYP-mediated drug metabolism as well as cytokine signaling and inflammatory response. CYP2A, CYP2B, CYP2F, and CYP2S are a CYP cluster on the same chromosome (Bui and Hankinson, 2009; Hoffman et al., 2001). The expressions of these CYPs and CYP2E have been found to be involved in the xenobiotic metabolism and synthesis of cholesterol, steroids, and lipids (Lewis et al., 2003; Thelen and Dressman, 2009; Zhang et al., 2002). In addition, the CYP2J2 and CYP2U1 inductions in the midbrain and heart could be associated with lipid metabolism and inflammatory responses because CYP2J2 can activate the nuclear peroxisome proliferator-activated receptor α, a controller of lipid metabolism and inflammatory responses (Xu et al., 2013). CYP2U1 also plays a physiological role in the fatty acid signaling processes and arachidonic acid signaling pathway modulation (Dhers et al., 2017; Siller et al., 2014). Hence, the upregulation of these CYPs in the extrahepatic tissues of PCB-exposed cats indicated that the PCB exposure may activate the lipid metabolism and inflammatory responses in cats. Thus, further studies on the adverse effects and toxicological risks related to the PCB exposure with the analysis of the lipid pathway and immune function in the feline hepatic and extrahepatic tissues are required.

The levels of PCB and OH-PCB in the liver

I also analyzed the PCB and OH-PCB levels in the liver samples of PCB-exposed cats. The average concentration (mean ± SD) of the total PCBs in the liver was 1270±2020 ng/g. In contrast the two purpose-bred mongrel dogs (age, 12–15 months; weight, 12–27 kg)

from the previous study (Korytko et al., 1999) had lower PCB levels (42.51–104.75 ng/g) in the liver, even though they were exposed to PCBs at almost the same levels (25 mg/kg) relative to our study. Comparing the PCB levels in the liver of cats and dogs, the higher residue of PCBs in the cat liver suggested that cats have a weaker metabolism for PCBs than dogs. However, the differences in the administration route and the exposure period of PCB between the dog study and our study may be responsible for the differences in the residual levels of PCB observed in the liver of dogs and cats. Among the OH-PCBs, 3–4 Cl-OH-PCBs were mainly presented in the cat liver. Especially, 4′OH-CB18 was the predominant hydroxylated metabolite in the liver of PCB-exposed cats, while CB18 had the lowest level. These results demonstrated that feline CYP, especially CYP1A1, CYP1A2, and CYP1B1, could mainly metabolize 3–4 Cl-PCBs and may have a low-affinity binding with 5–8 Cl-PCBs.

TEQ of DL-PCBs correlations with CYP1 mRNA expressions in the liver

Since DL-PCBs have been known as an inducer for the AHR-CYP1 signaling pathway (Girolami et al., 2015; Larigot et al., 2018), the relationship between the TEQ of DL-PCBs (CB77 and CB118) and CYP mRNA expression in the liver was investigated. The relative mRNA expression levels of CYP1A1 and CYP1B1 revealed the significant positive correlations with CB77 and the total TEQ. These results implied that these CYP expressions could be related to the DL-PCB level.

Toxic equivalency factor (TEF) indicates the degree of toxicity of dioxins and dioxin-like compounds (including PCBs) compared to the most toxic form of dioxin, 2,3,7,8-

tetrachlorodibenzo-p-dioxin, which is given a reference value of 1. The TEF values are determined based on in vitro and in vivo studies of dermal toxicity, immunotoxicity, reproductive deficits, teratogenicity, endocrine toxicity, and carcinogenicity promotion (Ahlborg et al., 1994; van den Berg et al., 2006). Comparing DL-PCBs in this study, CB77 presented the strongest correlations with the CYP1A1 and CYP1B1 mRNA expressions. Moreover, the higher TEF value of CB77 (0.0001) compared with that of CB118 (0.00003), suggests that CB77 may be the most potent inducer of CYP1A1 and CYP1B1 in the liver.

Results and Discussion: *The expressions of CYPs in the liver related to BDE-209 exposure in cats*

I analyzed the mRNA expressions of 17 CYP isoforms, including CYP1A1, CYP1A2, CYP1B1, CYP2A13, CYP2B11, CYP2C41, CYP2D6, CYP2E1, CYP2E2, CYP2F2, CYP2F5, CYP2J2, CYP2S1, CYP2U1, CYP3A12, CYP3A131 and CYP3A132, in the livers of the control and BDE-209 exposure cats, but hepatic CYP2B11 mRNA expression could not detect in this study. This finding similar to the previous studies (Khidkhan et al., 2019; Okamatsu et al., 2017b). A deficiency of CYP2B in the cat liver may relate to the potential toxicity risk of some drugs and pollutants, because CYP2B play a role in metabolism of 2%-10% of drugs (ex. cyclophosphamide, propofol and ketamine) (Hedrich et al., 2016) and environmental contaminants including 2,2′,4,4′-tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) in humans (Erratico et al., 2012; Feo et al., 2013).

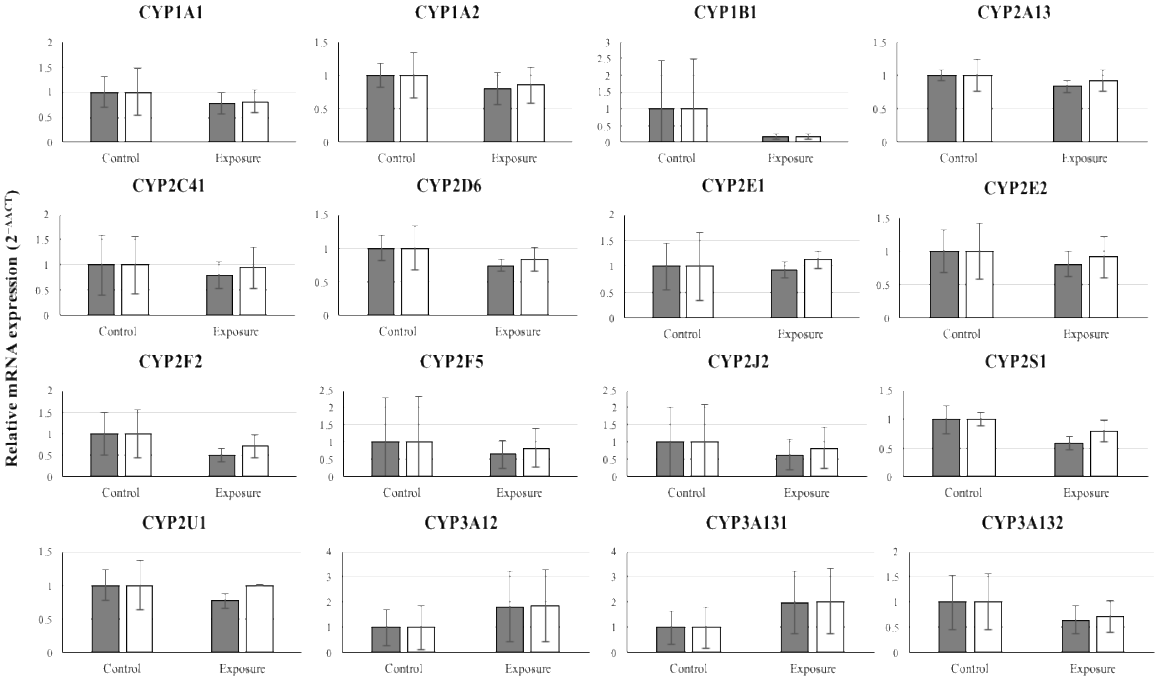

Fig. 8 Comparison of relative CYP mRNA expression (2−ΔΔCT, mean ± SD) in the liver of control and BDE-209 exposed cats (Reference genes: GAPDH [grey] and ACTB [white]).

The comparison of relative mRNA expressions of detected CYP isoforms in the livers between the control and BDE-209 exposure groups are given in the Fig. 8. Although the mRNA expressions of all CYP isoforms were not statistically different between control and exposure groups, CYP3A12 (1.8- to 1.9-fold) and CYP3A131 (2.0-fold) tended to be higher in the exposure group compared with those in the control group. I recently reported that CYP3A is dominant subfamily in the liver (Khidkhan et al., 2019) and it could play a major role in hepatic xenobiotic metabolism for cats (Honda et al., 2011; Khidkhan et al., 2019). However, the liver microsome in cats revealed lower CYP3A activity than in humans and

dogs and the relationship between molecular structure and metabolic activity is still largely unknown in cats (van Beusekom et al., 2010). Thus, the studies on feline CYP3A activity and the selectivity of CYP3A for BDE-209 metabolism are also necessary. CYP3A4 was suggested to be the specific enzyme responsible for the biotransformation of OH-PBDEs (3′-OH-BDE-7, 4′-OH-BDE-17 and 3-OH-BDE-47) in pig liver (Li et al., 2016). In rats, CYP3A1 was the most active in BDE-99 metabolism (400 pmol/min/mg protein) compared to BDE-47 (60 pmol/min/mg protein) (Erratico et al., 2011). Moreover, the statistical correlations among the hepatic expression levels of CYP1B1, CYP3A12 and CYP3A131 were noticed (Spearman's rank coefficients = 1, P = <0.0001) that indicated the trends of higher CYP3A12 and CYP3A131 expressions may strongly relate to the lower trend of CYP1B1 expression level in the BDE-209 exposed group. Comparing the control and exposure groups, the relative fold change of most CYP1 and CYP2 families ranged from 0.7 to 1, while the lowest relative fold change of CYP1B1 (0.2-fold) suggested that CYP1B1 mRNA expression was likely to be inhibited by BDE-209 (Fig. 8). Previous evidence revealed that CYP1B1 has been extremely active in the bioactivation of polycyclic aromatic hydrocarbons, such as tetrachlorodibenzo-*p*-dioxin, in mouse embryo fibroblast and human mammary epithelial cells (Alexander et al., 1997; Larsen et al., 1998). Also, our *in vivo* study presented the up-regulation of CYP1B1 mRNA expression in the liver (37.7-fold) and other tissues of cats exposed to PCBs compared to the control cats (Khidkhan et al., 2019). However, no such changes in CYP1B1 mRNA expression were noted in human mammary carcinoma cells exposed to low-dose PBDEs, including BDE-47, BDE-99, BDE-153, BDE-

183 or BDE-209 (Barber et al., 2006). In addition, the CYP1B1 mRNA down-regulation by BDE-209 has not been reported so far.

The studies on CYP expressions related to BDE-209 exposure are partly summarized and compared to our study in the Table 3. The up-regulations of CYP1A2 and CYP3A4 genes were observed in human hepatocytes exposed to BDE-209 for the periods of 24–72 h (Stapleton et al., 2009). In Sprague-Dawley rats, oral BDE-209 exposure (100 mg/kg) for three months could up-regulate CYP2B1 mRNA expression in the liver (Wang et al., 2010) and the protein expressions of CYP1A2, CYP2B1 and CYP3A1 were also induced by oral BDE-209 exposure (100-600 mg/kg) for one month (Lee et al., 2010), however, the sub-chronic BDE-209 exposure study (90 days) at a lower doses (10-50 mg/kg) was not found any inductions of CYP expression in the liver (Wang et al., 2012). The study of Sun et al (2020) suggested that BDE-209 could inhibit the pregnane X receptor (PXR) and constitutive androstane receptor (CAR) expression contributing to CYP3A suppression and impaired hepatic metabolic capacity in rat exposed to BDE-209 at the middle and high doses (50 and 500 mg/kg), but not at the low dose (5 mg/kg) (Sun et al., 2020). In addition to the differences in age and species, these studies implied that administered dose and exposure time may be important factors for BDE-209 induced CYP expression in the liver. Because of its large molecule, BDE-209 is poorly absorbed by oral route (less than 1% of the administered dose), slightly accumulated in the liver, and excreted almost completely through the feces (Costa and Giordano, 2011). In our study, BDE-209 was partially absorbed by the intestinal tract throughout the liver because it can be detected in the serum (420-550 ng/g) and liver (860-1,200 ng/g) (unpublished data). The debrominated metabolites observed in the liver indicate

BDE-209 could be metabolized by enzymes in the liver of cats, however, OH-PBDEs were not detected. These findings suggest that feline CYPs may not be major pathway for BDE-209 metabolism. Consequently, the orally administrated dose (7 mg/kg/week) in the present study could not induce CYP1-3 mRNA expressions in cats. The previous monitoring studies in pets suggested that OH-PBDEs in the blood of cats are derived from the CYP-dependent demethylation of naturally occurring methoxylated polybrominated diphenyl ethers (MeO-PBDEs) in cat foods, not from the hydroxylation of PBDEs (Mizukawa et al., 2013; Nomiyama et al., 2017). The extent of CYP expression is not involved in only toxic effects, but also connected the metabolism capacity for BDE-209 in each species. The study of *in vitro* biotransformation of BDE-209 using the liver of ring-billed gulls (*Larus delawarensis*) suggested that CYP-mediated metabolism is a minor metabolic pathway for BDE-209 (Chabot-Giguère et al., 2013). Therefore, the information on other phase I and II enzymes-mediated BDE-209 metabolism is also critical to understand the complete risks associated with BDE-209 exposure in cats.

Table 3 Comparison of the studies on CYP expressions related to BDE-209 exposure compared to this study.

Experiment	Stapleton et al., 2009	Lee et al., 2010	Wang et al., 2012	Sun et al., 2020	This study
Animal	Human hepatocyte	SD rat	SD rat	SD rat	Cat
Sex	Male and Female	Male	Male	Male	Male
Age	38-61 years old	10 days old	21 days old	42 days old	13-14 months old
Chemical	BDE-209	BDE-209	BDE-209	BDE-209	BDE-209
Dose	10 μM	100-600 mg/kg	10-50 mg/kg	5-500 mg/kg	7 mg/kg/week
Time of exposure	24-72 h	32 days	90 days	28 days	1 Year
Route of exposure	Direct incubation	Oral	Oral	Oral	Oral
CYP expression	CYP1A2 and CYP3A4 (up-regulation)	CYP1A2, CYP2B1 and CYP3A1 (up-regulation)	No significantly different	CYP3A1 and CYP3A2 (down-regulation)	No significantly different

Conclusions

Our study is the first extensive and statistical study to elucidate the various tissue distribution of the CYP mRNA expressions in cats. CYP3A was the dominant subfamily in the liver, but some CYP isoforms such as CYP1A1 and CYP2B11 were greatly expressed in the extrahepatic tissues. Our data showed that CYP1A1, CYP1A2 and CYP1B1 mRNA expressions could be clearly induced by PCBs and may be strongly induced by CB77 in several tissues. In addition, these isozymes may play a significant role in the metabolism of PCBs, particularly 3–4 Cl-PCBs, in the cat liver. However, the chronic exposure of BDE-209 could not induce CYP1–CYP3 mRNA expression in the liver of cats. Since the increased prevalence of organohalogen substances (including PCBs and PBDEs) in cats and household environment, the evidence and further studies on toxicokinetic process related to the potential health risk of these environmental contaminants are needed.

Chapter 3: Comparisons for chemical metabolism - the specific cytochrome P450 isoforms and cytochrome P450 activity between cats and other species

Abstract

Hydroxylated polychlorinated biphenyls (OH-PCBs), one of the toxic metabolites of PCBs, are primarily produced by cytochrome P450 (CYP) enzymes during the phase I reaction and their residue levels have been monitored in humans and domestic pets. Canine species such as the dog, fox, and raccoon dog showed a high proportion of highly Cl-OH-PCBs (6–8 Cl), whereas, in the cat, major components of OH-PCBs in the blood were lower Cl-OH-PCBs (3–5 Cl). I hypothesized that the interspecies differences between CYP expression in cats and dogs might be related to PCB metabolism. Neonicotinoid insecticides are used for agricultural and non-agricultural purposes worldwide. Cats and dogs are directly exposed to neonicotinoids in veterinary products and through environmental contamination. However, neonicotinoid CYP activities and metabolite compositions are unknown in most domesticated pet species. To be able to understand the interspecies differences among CYPs involved in PCB and neonicotinoid metabolism, the objectives of this study were to compare the production of OH-PCBs by CYP-mediated metabolism, to examine whether feline CYPs can metabolize high Cl-PCBs (7–8 Cl), and to reveal the differences in neonicotinoid metabolites and CYP activities among common pet species. The metabolic assay for 12 PCB (3–8 Cl) and highly Cl-PCBs (7–8 Cl) mixtures were conducted *in vitro* using hepatic microsomes of control cats, PCBs-exposed cats, control dogs, and PCBs-exposed dogs. The OH-PCB profiles between cats and dogs were similar for low Cl-OH-PCBs (3–5 Cl), especially 4′OH-CB18 as the major metabolite. These results, combined with *in silico* docking simulation, indicated that cat CYP3A and dog CYP3A/1A1 mainly contribute to the metabolism of PCBs, particularly PCB18. However, CYP1A1 in cats and CYP1A2/2B in

dogs may be minor players for the metabolism of some PCB congeners that led to their metabolites being formed in small amounts. Variations in neonicotinoid metabolism were found among species; enzyme kinetics indicated noticeably high V_{max}/K_m values in rats and humans in neonicotinoid metabolism, while the CYP activity in neonicotinoid metabolism was low in cats and dogs. The feline glucuronidation deficiency, together with our findings, suggested that neonicotinoids and PCBs were metabolized less in cats compared to other species. Therefore, the PCB contaminations in households and using drugs containing neonicotinoids in pets, especially cats, should be considered carefully in toxicology and veterinary medicine.

Keyword: PCBs, OH-PCBs, cytochrome P450, hepatic microsome, neonicotinoids, species variations, *in vitro* microsomal assay

Introduction

Cats and dogs are the most popular pets and great companions for humans. They have shared space with humans and are closely related to the indoor environment. Many studies have suggested that they have been useful sentinels for humans exposed to household chemicals (Ali et al., 2013; Dye et al., 2007; González-Gómez et al., 2018; Serpe et al., 2018; Venier and Hites, 2011). Concurrently, the development of knowledge in veterinary medicine and toxicology concerned with domestic dogs and cats has been expanded and has provided a better understanding of treatment issues (Pugliese et al., 2019; Pukay, 2000). For example, cats are known to be sensitive to acetaminophen and propofol due to deficiencies in uridine diphosphate glucuronosyltransferase (UGT) 1A6 and UGT1A9 (Court, 2013b), whereas chocolate poisoning in dogs is linked to decreasing theobromine metabolism with cytochrome P450 (CYP) 1A2 1117C>T polymorphism (Bates et al., 2015; Cortinovis and Caloni, 2016). These observations indicated that the variation in biotransformation plays an important role in determining chemical toxicity (Andersen, 1981; Baillie and Rettie, 2011). Recently, poisonous substances for cats and dogs have been very well-known (Cortinovis and Caloni, 2016; Kovalkovičová et al., 2009), but the understanding regarding the mechanism of toxicity of numerous agents is quite limited. Therefore, studies on xenobiotic metabolism in cats and dogs are essential with respect to medication and toxicology.

The metabolism of xenobiotics via xenobiotic-metabolizing enzymes (XMEs) has been categorized into phase I (functionalization) and phase II (conjugation) reactions (Nebert and Dalton, 2006). Hepatocytes primarily produce several XMEs including CYP, which

includes 70–80% of all phase I XMEs (Evans and Relling, 1999; Nebert and Dalton, 2006; Zanger and Schwab, 2013). CYP is a big family of hemoproteins that function as monooxygenases. Among CYP families, CYP1–3 are the most prominent families that play a key role in the metabolism of drugs and environmental pollutants (Evans and Relling, 1999; Zanger and Schwab, 2013). Species variations and genetic polymorphisms in CYP genes can affect the function of enzymes. Our previous study found that CYP3A mRNA expression was dominant in cat liver (Khidkhan et al., 2019), while CYP2 was dominant in dog liver, including CYP2A, CYP2D, and CYP2E (Martinez et al., 2013). These CYPs can be induced or inhibited by numerous substances, and identifying individual CYP isoforms responsible for the metabolism of these chemicals will be essential for predicting potentially toxic effects and treatment failures (Evans and Relling, 1999; Graham and Lake, 2008).

Polychlorinated biphenyls (PCBs) are a group of synthetic organic chemicals consisting of 209 individual PCB congeners, which differ in a pattern of chlorine substitution (Beyer and Biziuk, 2009). They have been recognized as one of the most hazardous persistent contaminants; their toxicities related to human diseases such as cancer, endocrine disruption, immune dysfunction, and neurodevelopmental toxicity have been documented (Boersma and Lanting, 2000; Donat-Vargas et al., 2019). Although PCBs were banned in the late 1970s worldwide, they have been continuously found in the environment (Beyer and Biziuk, 2009). Among 209 congeners, a small number of tri- to octa-chlorinated (3–8Cl) PCBs are more persistent and have been generally quantified in house dust and outdoor environments (Ali et al., 2018; Chandra Yadav et al., 2019; Gonzalez Sagrario Mde et al., 2002; Rauert et al., 2018; Wang et al., 2019). These PCB congeners can be metabolized into hydroxylated PCBs

(OH-PCBs) by CYP enzymes in the liver, which are highly toxic metabolites that have been routinely monitored in mammals, including domestic cats and dogs (Ali et al., 2013; Mizukawa et al., 2013). Since OH-PCBs are structurally similar to thyroid hormones (TH), they could disturb systems that regulate the hypothalamic-pituitary-thyroid axis (Amano et al., 2010; Miyazaki et al., 2004). Several monitoring studies have suggested that PCBs exposure contributes to the feline hyperthyroidism (Peterson, 2012; Walter et al., 2017), whereas PCBs in serum were not significantly associated with hypothyroxinemia in dogs (Lau et al., 2017). Recently, an *in vivo* study revealed that TH levels did not change in PCB-exposed cats, whereas total T4 and T3 levels decreased and free T4 levels increased in PCB-exposed dogs (Takaguchi et al., 2019). In addition, cats and dogs have revealed interspecies differences in the OH-PCBs accumulated in the blood (Mizukawa et al., 2013); cats presented a high composition of low Cl-OH-PCBs (3–5 Cl), whereas, in dogs, most OH-PCBs detected in the blood were high Cl-OH-PCBs (6–8 Cl). These different effects on TH and OH-PCB levels in cats and dogs could be due to PCBs interfering with TH metabolism (Takaguchi et al., 2019) as well as the biotransformation capacity of PCBs (Mizukawa et al., 2013). Therefore, studies focusing on PCB metabolism in cats and dogs are of critical importance. I hypothesized that the species-specific differences in CYP expressions and functions between cats and dogs may be related to PCB metabolism.

Neonicotinoids, such as imidacloprid, acetamiprid, and clothianidin, belong to a class of neuroactive insecticides linked to the activation of post-synaptic nicotinic acetylcholine receptors (nAChRs) that are highly selective towards some insect species (Casida, 2018; Sheets et al., 2016). The chemical structure of neonicotinoids varies by generation; the first-

generation neonicotinoids (imidacloprid, nitenpyram, thiacloprid, and acetamiprid) share a chloropyridine ring, whereas the core structure of second-generation neonicotinoids (thiamethoxam and clothianidin) has a chlorothiazole group (Thompson et al., 2020). Imidacloprid is one of the most common veterinary insecticides for eliminating fleas in cats and dogs, while acetamiprid and clothianidin are typical neonicotinoids used to control insects and pests in agricultural, commercial, and residential environments worldwide (Mehlhorn et al., 2001; Simon-Delso et al., 2015; Vo et al., 2010). Non-target vertebrates such as humans, pets, and wildlife can be unintentionally exposed to these neonicotinoid residues in the environments leading to toxicities and adverse effects that have been reported globally (Gibbons et al., 2015; Sheets et al., 2016; Simon-Delso et al., 2015). Neonicotinoids can be absorbed by the skin (7.9%-11.4%) and are efficiently absorbed through the intestine of humans and animals, causing neurotoxicity, hepatoxicity, and impaired immune function (Aggarwal et al., 2014; Li et al., 2012; Simon-Delso et al., 2015; Thompson et al., 2020). Numerous studies have indicated the toxicities related to the metabolism of neonicotinoids (Casida, 2011; Ford and Casida, 2006; Shi et al., 2009; Thompson et al., 2020), highlighting the need for a more thorough understanding of species differences in the metabolism of neonicotinoids for phase I and phase II biotransformation. The metabolism of neonicotinoids has been most commonly studied in mice, rats, and humans, and studies have found that the formation of many neonicotinoid metabolites is mediated by CYP (Schulz-Jander and Casida, 2002; Thompson et al., 2020). The CYP3 and CYP2 families are responsible for imidacloprid and clothianidin metabolism in humans (Casida, 2011; Schulz-Jander and Casida, 2002). However, the role of CYP in neonicotinoid metabolism in vertebrate species or domestic pets,

such as cats and dogs, is not well known. Moreover, studies of neonicotinoid toxicities in vertebrates suggest that there are toxicological risks associated with some neonicotinoid metabolites; understanding the metabolism of neonicotinoids is critical to characterizing potential health risks in each species (Casida, 2011; Thompson et al., 2020). To our knowledge, no information is available on the interspecies differences in the role of CYP in neonicotinoid metabolism or the variation in metabolite composition among species.

Due to the lack of information on CYP-mediated metabolism of neonicotinoids and PCBs in pets, especially domestic cats, this *in vitro* study aims to: investigate the interspecies differences in CYPs involved in PCB metabolism between cats and dogs; to determine whether feline CYPs can metabolize highly Cl-PCBs (7–8 Cl) by comparing the production of OH-PCBs and estimation pathways of CYP-mediated PCB metabolism using *in vitro* metabolism of PCBs (with substrates of mixtures of 3–8Cl PCBs and 7–8Cl PCBs) and *in silico* docking simulation; to examine the interspecies differences in neonicotinoid metabolite formation following CYP metabolism; and to elucidate CYP activities in the metabolism of first-generation (imidacloprid and acetamiprid) and second-generation (clothianidin) neonicotinoids in cats, dogs, rats, and humans.

Materials and Methods

Chemicals

For PCB metabolism assay, PCB18, PCB28, PCB70, PCB77, PCB99, PCB101, PCB118, PCB138, PCB153, PCB180, PCB187, and PCB202 (purity: ≥99.5%) were purchased from AccuStandard Inc. (CT, USA). Magnesium chloride ($MgCl_2$), glucose 6-phosphate (G6P), glucose 6-phosphate dehydrogenase (G6PDH), beta-nicotinamide adenine dinucleotide phosphate (β-NADPH), potassium hydroxide (KOH), methyl tert-butyl ether, silica gel (Wako-gel S1), methanol, decane, sodium sulfate anhydrous, sulfuric acid, hexane, and trimethylsilyl-diazomethane were purchased from Wako Pure Chemical Ind. (Osaka, Japan). Dichloromethane and ethanol were obtained from Kanto Chemical Co., Inc. (Tokyo, Japan), and DMSO was purchased from Nacalai Tesque (Tokyo, Japan). $^{13}C_{12}$-labeled internal standards of 4OH-CB29, 4'OH-CB61, 4'OH-CB79, 4OH-CB107, 4OH-CB120, 4OH-CB146, 4OH-CB157, 4OH-CB172, and 4-OH-CB187 (purity: ≥99%) were obtained from Wellington Laboratories Inc. (Ontario, Canada). The BCA protein assay kit and Pierce ECL Western Blotting Substrate were obtained from Thermo Fisher Scientific (MA, USA). For the specific antibodies, the mouse anti-rat CYP1A1/CYP1A2 and rabbit anti-rat CYP1B1 were purchased from Thermo Fisher Scientific (IL, USA), goat anti-rat CYB2B1 was obtained from Daiichi Pure Chemicals Co., Ltd (Tokyo, Japan), rabbit anti-human CYP3A4 was purchased from Sigma-Aldrich (MO, USA), and rabbit anti-human beta-actin was purchased from Abcam (Tokyo, Japan). The secondary antibodies, including the donkey anti-goat IgG, goat anti-rabbit IgG, and goat anti-mouse IgG conjugated with horseradish peroxidase, were obtained from Santa Cruz Biotechnology (CA, USA).

Samples for in vitro PCBs metabolism assay

Liver samples of cats and dogs were obtained from the previous experiment (Takaguchi et al., 2019). Briefly, the exposure tests for eight male cats (24–28 months old, 3–5 kg, *Felis catus*) were performed in accordance with the guideline of the Association for Assessment and Accreditation of Laboratory Animal Care International at the Faculty of Veterinary Medicine, Hokkaido University, Japan (approval number 14-0054 and 14015) and for six male beagle dogs (5–7 months old, 6–7 kg, *Canis lupus familiaris*) at the Korea Institute of Toxicology, Korea (approval number 13027). After two weeks of acclimation, the animals were divided into two groups: control (cat: n = 4, dog: n = 3) and exposure (cat: n = 4, dog: n = 3). The animals were intraperitoneally injected, on one occasion, with corn oil and a mixture of twelve PCB congeners (PCB18, PCB28, PCB70, PCB77, PCB99, PCB101, PCB118, PCB138, PCB153, PCB180, PCB187, and PCB202) in corn oil at a dose of 0.5 mg (each congener)/kg (BW) in the control and exposure groups, respectively. After a 120-hour exposure period, the cats and dogs were anesthetized with pentobarbital and euthanized using KCl injection. Liver tissues were immediately collected in liquid nitrogen and stored at –80 °C until further analysis.

Microsome preparation for PCB metabolism assay

The microsome fraction was prepared using the method described by Omura and Sato (1964), with some modifications. Livers were homogenized three times with 0.1 M potassium phosphate buffer (KPB, pH 7.4). The homogenates were centrifuged at 9,000 rpm at 4 °C for 20 min. The supernatants were filtered with sterile gauze and centrifuged twice at

34,000 rpm at 4 °C for 60 min. The microsomal pellets were resuspended with 0.1 M KPB and stored at −80 °C until use. The protein concentration of the microsomes fraction was measured using the BCA protein assay kit. The CYP content was determined by the previous method (Omura and Sato, 1964).

In vitro PCB metabolism assay

The PCB metabolism assay using liver microsomes was conducted using the method described by previous study (Wu et al., 2014) with slight modifications. The reaction mixture included 0.1 M KPB (pH 7.4), $MgCl_2$ (final concentration, 3 mM), and G6P (final concentration, 5 mM) was mixed with the liver microsome (CYP concentration, 200 pmol). The 12 PCB mixtures (3-8Cl; PCB18, PCB28, PCB70, PCB77, PCB99, PCB101, PCB118, PCB138, PCB153, PCB180, PCB187, and PCB202) or highly Cl-PCB mixtures (7-8Cl; PCB180, PCB187, and PCB202) in DMSO was added with a final concentration of 1 ppm and preincubated at 37 °C for 5 min. A mixture of G6PDH (final concentration, 2 IU/mL) and β-NADPH (final concentration, 0.5 mM) was applied to each sample to start the reaction. After 2 hours in a shaking water bath, 1 M KOH in 50% ethanol/water was added to stop the reaction. All assays were carried out in duplicate for each sample. Negative controls (the reaction mixture without PCBs) were performed in parallel.

Chemical analysis and quality assurance

OH-PCBs in the reaction mixture were analyzed using the method described by Nomiyama et al. (2010). Briefly, $^{13}C_{12}$-labeled OH-PCBs were placed into each reaction sample (2 ml). After adding hexane and centrifugation, the supernatant containing PCBs was

removed to a new tube. The aqueous layer containing OH-PCBs was adjusted to pH 1–2 using sulfuric acid, extracted twice using 50% methyl tert-butyl ether/hexane, and then derivatized to methylated compounds (MeO-PCBs) overnight using trimethylsilyl-diazomethane. The derivatized solution was passed through the activated silica gel column, eluted with 10% dichloromethane/hexane, and then concentrated. OH-PCBs were identified and quantified using a gas chromatograph (6890 series, Agilent Technologies) coupled to a high-resolution (>10,000) mass spectrometer (JMS-800D, JEOL).

The target OH-PCBs were measured from the corresponding $^{13}C_{12}$-internal standards using the isotope dilution method (Eguchi et al., 2014; Nomiyama et al., 2010). A procedural blank was considered in each batch (8 samples) to identify any possible contamination from glassware and solvents. The recovery of samples was 24-46% for OH-PCBs. However, these reaction mixtures with matrix effects were corrected for the potential influence of foreign substances by $^{13}C_{12}$-label internal standards in this study.

Western blotting for liver microsomes of cats and dogs

Protein identification for CYP1A1/1A2, CYP1B1, CYP2B and CYP3A was performed using western blotting (Laemmli, 1970). Liver microsomes containing 10 µg of protein were applied to 10% sodium dodecyl sulfate-polyacrylamide gels and separated by electrophoresis using a Mini-PROTEAN Tetra cell (Bio-Rad, CA, USA). The proteins were then transferred to nitrocellulose membranes (Toyo Roshi Kaisha Ltd., Tokyo, Japan). Membranes were subsequently incubated in 5% skim milk at 25 °C for 1 hour to block non-specific binding. They were then immunoblotted with specific antibodies followed by

incubation with secondary antibodies. A specific band was detected using Pierce ECL Western Blotting Substrate. The density of the specific band was analyzed using the image analysis program Image J (National Institutes of Health, MD, USA).

In silico analysis for PCBs and CYP isoforms estimation pathways

All *in silico* analyses were carried out using the Molecular Operating Environment (MOE) program (Chemical Computing Group, Montreal, Canada) according to the previous methods (Hirakawa et al., 2018; Yoo et al., 2015). To construct the homology models of heme-containing CYP proteins of the dog and cat, the following structural data of CYP1, 2, and 3 isozymes were taken from the Protein Data Bank (http://www.rcsb.org) and were used as templates: human CYP1A1 (PDB code: 4I8V), human CYP1A2 (PDB code: 2HI4), human CYP1B1 (PDB code: 3PMO), rabbit CYP2B11 (PDB code: 1SUO and 1DT6), human CYP3A12 (PDB code: 2V0M and 1TQN), and human CYP3A132 (PDB code: 2V0M and 1TQN). These structural data have been used in other *in silico* docking studies (Handa et al., 2013; Lautier et al., 2016; Yoo et al., 2015). All crystallographic water molecules were eliminated from the CYP structures. The 3D structures of human and rabbit CYPs were optimized using an Amber10:EHT force field after adding hydrogen atoms.

Molecular docking simulations were performed for the binding of CB18, CB28, CB77, CB99, CB101, and CB187 congeners to dog and cat CYP homology models using ASEDock (Ryoka Systems Inc., Tokyo, Japan) according to the previous methodology (Goto et al., 2008). Prior to the ASEDock analysis, structures of PCBs were constructed and their energies were minimized using Rebuild3D with the Amber10:EHT force field in the MOE.

A total of 500 conformations were generated for each PCB congener using LowMode MD method. The parameters used for the refinement step were as follows: a cutoff value of 4.5, an RMS gradient of 10, and an energy threshold of 500. The energy of the PCB-CYP complex was refined using PFROSST of MOE under limited conditions in which the backbones of amino acid residues were tethered and the side chains of amino acid residues were unconfined.

I selected the pose with the highest docking score among the results of CYP-PCB docking poses required to produce hydroxylated PCB metabolites that were actually detected by chemical analyses, showing < 6.0 Å distance of the heme Fe-C in the biphenyl and < 0 of the docking energy value (data summarized in Table 1).

Sample and microsome preparation for in vitro neonicotinoid metabolism assay

Table 2 details the samples used in this study. Human microsomes were purchased from Celsis In Vitro Inc. (MD, USA). Cat and dog liver samples were obtained from vehicle control animals in the previous studies performed at the Faculty of Veterinary Medicine, Hokkaido University, Japan (approval number 14-0054 and 14015) and at the Korea Institute of Toxicology, Korea (approval number 13027), respectively (Khidkhan et al., 2019; Takaguchi et al., 2019). Sprague Dawley (SD) rats (7-week-old) were acquired from Sankyo Lab Service Co., Ltd. (Hokkaido, Japan). Microsomal fractions of rats, dogs, and cats were prepared and modified using the method described by Omura and Sato (1964). Livers were homogenized with three volumes of 0.1 M potassium phosphate buffer (KPB, pH 7.4). The homogenates were centrifuged at 9,000 rpm at 4 °C for 20 min. The supernatants were filtered with sterile gauze and centrifuged twice at 34,000 rpm at 4 °C for 60 min. Microsomal pellets

were resuspended in 0.1 M KPB and stored at -80 °C until use. The protein concentrations of microsomal fractions were measured using the BCA protein assay kit (Thermo Fisher Scientific, IL, USA), and CYP concentration was determined using a previously reported method (Omura and Sato, 1964).

Table 2 Details of animals used to obtain liver microsomes

Species	**SD Rat**	**Dog (Beagle)**	**Cat**	**Human**
Scientific name	*Rattus rattus*	*Canis familiaris*	*Felis catus*	*Homo sapiens*
Age	7 weeks	5-7 months	24-28 months	19-77 years
Sex	Male	Male	Male	Male and Female
Number of samples	4	3	4	10
Sample procurement	Purchased	Previous study (Takaguchi et al., 2019)	Previous study (Khidkhan et al., 2019)	Purchased

In vitro CYP metabolism of neonicotinoids

Neonicotinoid metabolism assay using liver microsomes was conducted using the methods described in the previous report (Wu et al., 2014). The reaction mixture included 0.1 M KPB (pH 7.4), $MgCl_2$ (final concentration 3 mM), and G6P (final concentration 5 mM) mixed with pooled hepatic microsomes (final protein concentration 5 mg/mL). Imidacloprid (Kanto Chemical Co., Inc., Tokyo, Japan), clothianidin (Wako Pure Chemical Co., Osaka, Japan), or acetamiprid (Cosmo Bio Co., Ltd., Tokyo, Japan) in 3% MeOH were added (final substrate concentration of 10, 25, 50, 100, 200, and 400 μM) and pre-incubated at 37 °C for 5 min. A mixture of G6PDH (final concentration 2 IU/mL) and β-NADPH (final

concentration 0.5 mM) was added to each sample to start the reaction. After 30 minutes in a shaking water bath, 100% MeOH was added to stop the reaction. Reaction samples were then placed on ice for 15 min before centrifugation at 15,000 g for 10 min and filtered using the GL Chromato Disk sample filter (pore size 0.2 µM; GL Sciences, Tokyo, Japan). All assays were performed in duplicate for each sample. The appropriate negative controls (the reaction mixture without substrate) were used.

Chemical analysis for neonicotinoid metabolites

A liquid chromatography mass spectrometer (6495 triple quad LC/MS; Agilent Technologies, Santa Clara, CA, USA) equipped with a 1.7 µm Biphenyl 100 A LC column (2.1*150 mm; Kinetex, Phenomenex Inc, Torrance, CO, USA) was used to quantify the target metabolites of imidacloprid, clothianidin and acetamiprid. The target neonicotinoid metabolites were purchased from Sigma-Aldrich Co. LLC. (Darmstadt, Germany) or synthesized at Toho University (Chiba, Japan). Detection methods followed those of previous studies (Ichikawa et al., 2019; Ikenaka et al., 2019). For all analyses, mobile phase A consisted of 0.1% formic acid + 10 mM ammonium acetate in distilled water and mobile phase B consisted of 0.1% formic acid + 10 mM ammonium acetate in 100% MeOH. An injection volume of 5 µL, a flow rate of 0.35 mL/min, and a column temperature of 60 ^{o}C was used for all experiments. The limit of quantification was defined as the sufficient concentration of the target compounds to produce a signal to noise ratio (S/N) higher than 9 under the lowest calibration point (0.5 ppb for imidacloprid and clothianidin; 0.05 ppb for acetamiprid).

Data analyses of PCB metabolism assay and kinetics of neonicotinoids

Results are shown as average ± standard deviation (SD). The levels of OH-PCB congeners were tested for normality using the Shapiro-Wilk test and checked for homogeneity of variance using Levene's test. Wilcoxon test and Student's *t*-test were performed to determine the significant differences between the control and exposure groups using JMP Pro 13 (SAS Institute, USA). Total OH-PCB levels of the control and exposure groups in cats and dogs were compared via Tukey-Kramer test.

All kinetic parameters (including maximum velocity (V_{max}), Michaelis-Menten constants (K_m) and V_{max}/K_m ratio) were calculated using the Michaelis-Menten equation in GraphPad Prism version 8.0 for Windows (GraphPad Software, CA, USA). Statistical analyses were performed using JMP Pro 13 (SAS Institute, NC, USA). Tukey's HSD test was performed to compare the V_{max}/K_m among species. P-value < 0.05 was considered statistically significant in all analyses.

Results: *Comparison of CYP-mediated PCB metabolism in cats and dogs*

Levels of OH-PCBs in the reaction mixtures

The OH-PCBs congeners were measured after conducting *in vitro* PCB metabolism assay. The homologous compositions of OH-PCBs (3–8Cl OH-PCB) and proportions of OH-PCB congeners detected in the reaction mixtures are shown in Fig. 1 and 2, respectively. Using the 12 PCB mixture as a substrate, 3Cl OH-PCB, especially 4′OH-CB18, were the major metabolites in the control and exposure groups of cats as well as the control group of dogs. On the other hand, 4Cl OH-PCB, mainly 4′OH-CB79, was more easily detected in the exposure group of dogs, followed by 3Cl OH-PCB and 5Cl OH-PCB. The total OH-PCB level in the control group was significantly higher than the exposure group using dog liver microsomes. When the highly Cl-PCB mixture was applied as the substrate, no significant difference was found in the total OH-PCB levels between the control and exposure groups of cats and dogs. The 6–8Cl OH-PCBs (3OH-CB153, 4OH-CB178, 4OH-CB187 and 4OH-CB202) and a negligible amount of 7Cl OH-PCBs (4OH-CB187 and 4OH-CB202) were found in the control and exposure groups of dogs and cats, respectively. However, total OH-PCB levels in the dog control and exposure groups were statistically higher than those in cats using both substrates (12 PCB mixture and highly Cl-PCBs mixture).

Figs. 3 and 4 show the comparisons of OH-PCB congeners that were significantly different between control and exposure groups in cats and dogs. Comparisons between the cat control and exposure groups indicated that the level of 4′OH-CB18 was significantly lower in the exposure group, whereas 4′OH-CB61 and 4′OH-CB79 levels were statistically

higher in the exposure group, using the substrate of 12 PCB mixture. Reaction mixtures between dog liver microsomes and the mixed 12 PCBs, the 4′OH-CB18 and 4OH-CB97 levels were significantly lower in the exposure group than in the control group, 4′OH-CB25/26/4OH-CB31, 4′OH-CB35, 4′OH-CB61, 4OH-CB70, 4′OH-CB79, and 3OH-CB101 were significantly higher in the exposure dogs compare to those in the control dogs. The small amounts of OH-PCBs were observed after using the substrate of the 7–8Cl PCB mixtures in both cats and dogs. Comparisons between the control and exposure groups in each species, the 3OH-CB101 and 4OH-CB187 levels were significantly higher in the cat exposure group, while the 4OH-CB70, 3OH-CB101, and 4OH-CB101 levels were significantly higher in the dog exposure group.

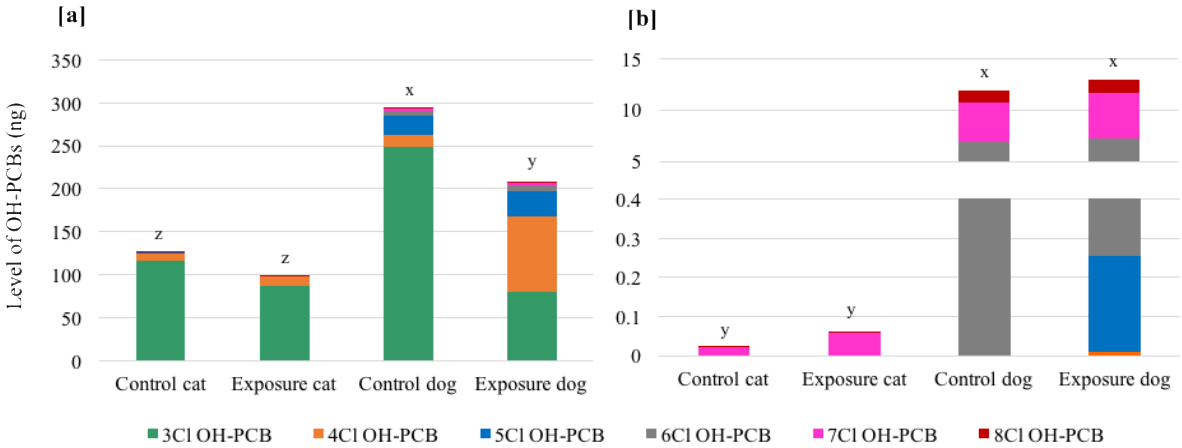

Fig. 1 Average total OH-PCB levels and homologous compositions of OH-PCBs (3-8Cl OH-PCB) in the reaction mixtures (substrate: a mixture of 3-8Cl PCBs [a] and mixture of 7-8Cl PCBs [b]) using liver microsomes of the control and exposure groups in cats and dogs. Different characters (x, y, z) indicate statistically significant differences in total OH-PCB levels (Tukey-Kramer test, $P < 0.05$).

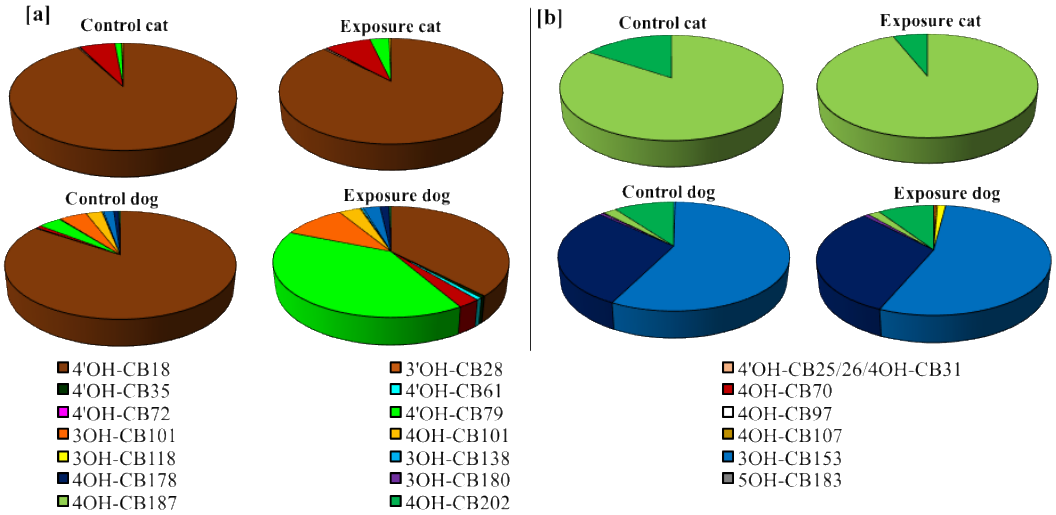

Fig. 2 Proportions of OH-PCB congeners detected in the reaction mixtures (substrate: a mixture of 3-8Cl PCBs [a] and mixture of 7-8Cl PCBs [b]) using liver microsomes of control and exposure groups in cats and dogs.

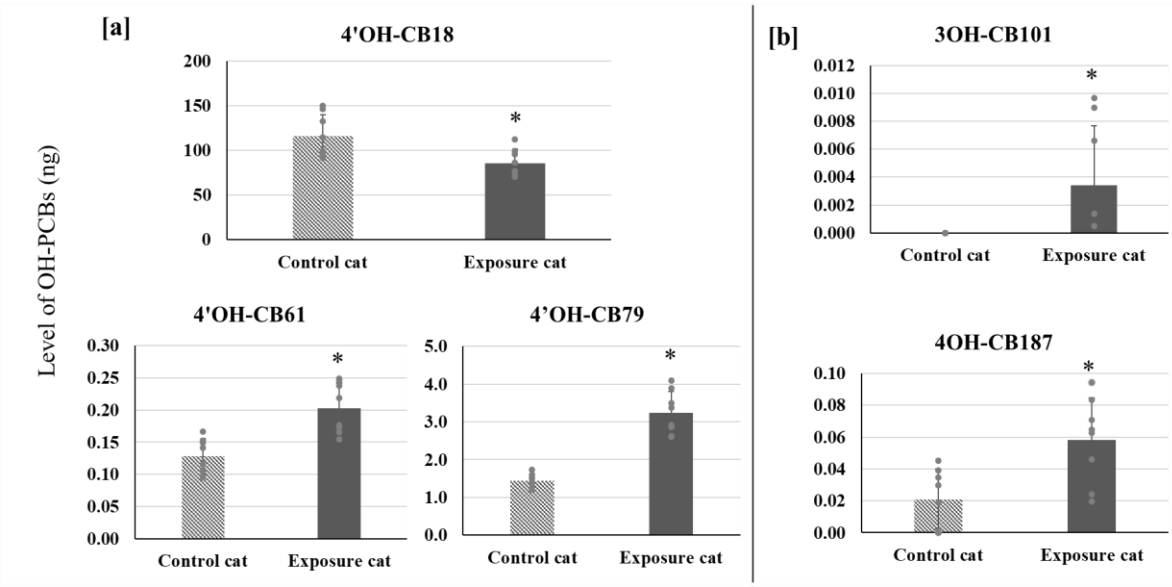

Fig. 3 Comparison of OH-PCB congeners in the reaction mixtures (substrate: a mixture of 3-8Cl PCBs [a] and mixture of 7-8Cl PCBs [b]) using cat liver microsomes between the control and exposure groups (Student's t-test, *: $P < 0.05$).

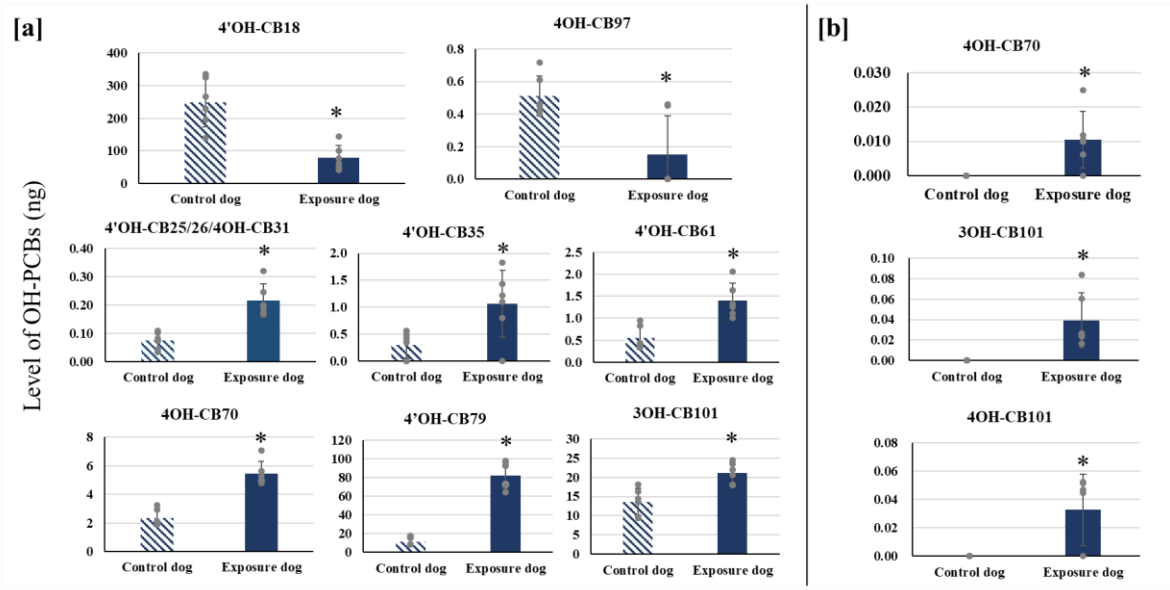

Fig. 4 Comparison of OH-PCB congeners in the reaction mixtures (substrate: a mixture of 3-8Cl PCBs [a] and mixture of 7-8Cl PCBs [b]) using dog liver microsomes between the control and exposure groups (Student's *t*-test, *: $P < 0.05$).

Protein expression levels of hepatic CYP

The protein expression of CYP1A1/1A2, CYP1B1, CYP2B, and CYP3A in the hepatic microsomes of the control and exposure groups of cats and dogs is shown in Figs. 5 and 6. The protein expression levels of CYP1A1/1A2 and CYP2B were significantly elevated in the liver microsomes of the exposure group of dogs compared to the control. For cats, only the level of CYP1A1/1A2 protein was statically higher in the exposure group than the control group. No significant differences in CYP3A protein expression were observed in hepatic microsomes between the control and exposure groups of cats and dogs. In addition, the

protein expression of CYP2B and CYP1B1 could not be detected in the liver microsome fractions of cats and dogs, respectively.

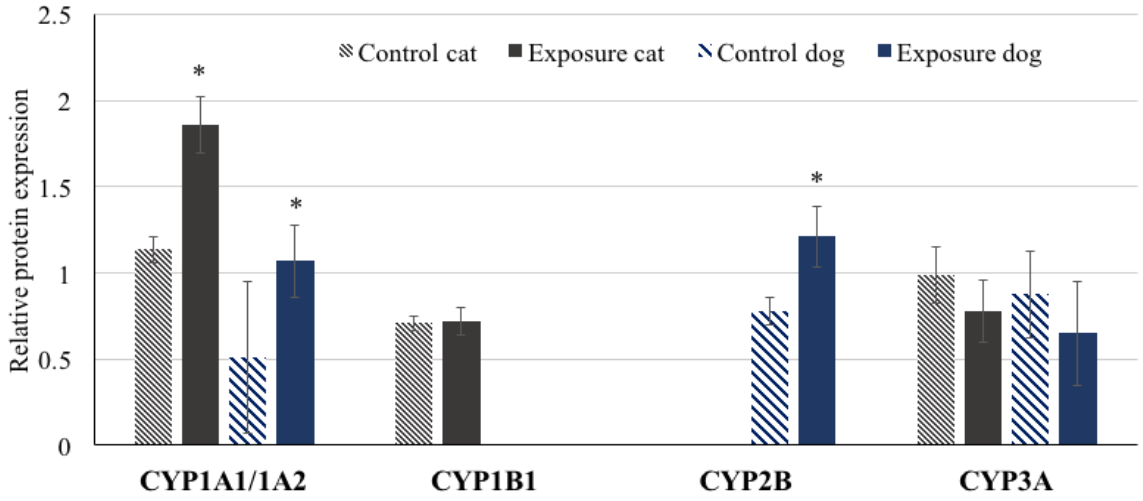

Fig. 5 Average protein expression levels of CYP1A1/1A2, CYP1B1, CYP2B, and CYP3A in the liver microsomes of control and exposure groups in cats and dogs. Asterisks (*) indicate statistically significant difference between control and exposure groups (Wilcoxon test, $P < 0.05$).

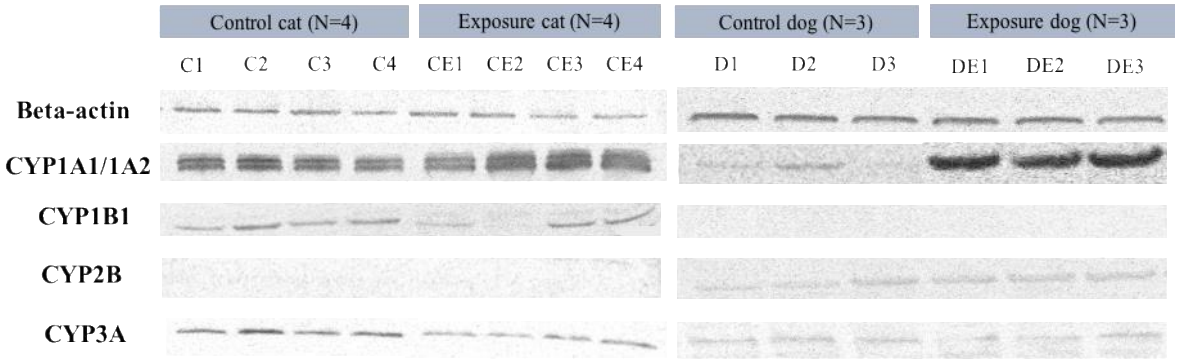

Fig. 6 Western blotting of CYP1A1/1A2, CYP1B1, CYP2B, and CYP3A of control cats (C1-C4), exposed cats (CE1-CE4), control dogs (D1-D3), and exposed dogs (DE1-DE3)

Investigation of CYP dependent metabolic potential of PCB congeners

The results of *in silico* docking simulation are shown in Table 3. I selected the PCB congeners, including PCB18, PCB28, PCB77, PCB99, PCB101, and PCB187, which have been suggested as substrates of detected metabolites (4'OH-CB18, 4'OH-CB25, 4'OH-CB79, 4OH-CB97, 3OH-CB101, and 4OH-CB187/5OH-CB183). CYP isoforms in cat and dog were chosen according to the results of the *in vitro* CYP metabolism of PCBs in this study together with the findings of PCBs-induced feline CYP mRNA expression from our previous study (Khidkhan et al., 2019). In cats, docking simulation results indicated that CYP3A132 was the most important for the metabolism of PCB18, PCB28, PCB77, PCB99, PCB101, and PCB187. Feline CYP1A1 contributed less to the metabolism of PCB28 and PCB77, and both CYP1A2 and CYP1B1 were not responsible for the metabolism of these congeners. On the other hand, in dogs, CYP3A12 and CYP1A1 were major isozymes for the metabolism of all selected PCB congeners, CYP1A2 and CYP2B11 contributed less to the metabolism of PCB28, and PCB77 and PCB187.

Table 1 Target-template sequence similarity

	Dog						Cat				
	CYP1A1 (4I8V)	**CYP1A2 (2HI4)**	**CYP2B11 (1SUO)**	**CYP2B11 (1DT6)**	**CYP3A12 (2V0M)**	**CYP3A12 (1TQN)**	**CYP1A1 (4I8V)**	**CYP1A2 (2HI4)**	**CYP1B1 (3PMO)**	**CYP3A132 (2V0M)**	**CYP3A132 (1TQN)**
Sequence Homology (%)	81.6	82.8	79.1	51.6	76.9	78.0	80.2	80.5	84.3	76.4	76.9

Table 3 *In silico* docking simulation in each pair of CYPs and PCB congeners (E: docking energy (kcal/mol), D: distance: distance (Å) between heme iron and target carbon of PCB where hydroxylation was predicted to takes place.)

Predicted reaction	**Target carbon in PCB**	**Dog**								**Cat**							
		CYP1A1		**CYP1A2**		**CYP2B11**		**CYP3A12**		**CYP1A1**		**CYP1A2**		**CYP1B1**		**CYP3A132**	
		D	**E**	**D**	**E**	**D**	**E**	**D**	**E**	**D**	**E**	**D**	**E**	**D**	**E**	**D**	**E**
PCB18 -> 4'OH-CB18	4'	4.75	-27.9	-	-	-	-	4.57	-28.3	-	-	-	-	-	-	4.30	-34.2
PCB28 -> 4'OH-CB25	3'#	5.05	-30.9	5.16	-11.7	-	-	4.78	-31.0	3.21	-5.17	-	-	-	-	5.05	-29.9
PCB77 -> 4'OH-CB79	5#	5.78	-35.0	-	-	4.96	-6.49	5.32	-36.8	3.23	-0.73	-	-	-	-	3.47	-34.3
PCB99 -> 4'OH-CB97	3'#	4.85	-31.9	-	-	-	-	-	-	-	-	-	-	-	-	5.97	-32.1
PCB101 -> 3OH-CB101	3	-	-	-	-	-	-	4.86	-38.0	-	-	-	-	-	-	5.69	-33.0
PCB187 -> 4OH-CB187/ 5OH-CB183	4#	-	-	-	-	5.9	-21.6	3.36	-42.4	-	-	-	-	-	-	5.29	-34.7

#: predicted NIH shift

Discussion: *Comparison of CYP-mediated PCB metabolism in cats and dogs*

Interspecies differences in PCB metabolism

In the present study, I evaluated the ability of hepatic microsomes to produce 3–8Cl PCB metabolites and compared the interspecies differences in CYP-mediated PCB metabolism between cats and dogs. The composition of OH-PCB congeners and total OH-PCB levels produced by cat and dog microsomes indicated that cat microsomes had a low capacity for metabolizing the mixtures of 3–8Cl PCBs and 7–8Cl PCBs compared to the dog microsomes. Furthermore, analyses of the species-specified hepatic metabolism of PCB51 and PCB102 revealed that dogs can produce higher OH-PCBs levels than hamsters, rabbits, rats, guinea pigs, and monkeys (Uwimana et al., 2017). Our results that focused on phase I metabolism suggested that 4′OH-CB18 is the major OH-PCB metabolite in both dogs and cats. The OH-PCB profiles after *in vitro* metabolism of the mixtures of 3–8Cl PCBs and 7–8Cl PCBs revealed that lower Cl-PCBs (3-5Cl) are metabolized easier than higher Cl-OH-PCBs (6–8Cl). Other studies suggested that the fewer the number of chlorine atoms on biphenyl as well as the meta- and para- positions of the biphenyl ring, results in a faster metabolism by enhancing CYP-mediated transformation (Grimm et al., 2015; Mills et al., 1985).

Comparisons of the PCB metabolism between cats and dogs indicated that the cats could have a very low capacity for phase II metabolism of PCBs because the most detected OH-PCBs (3-5Cl OH-PCBs) in the blood of cats in the previous study (Mizukawa et al., 2013) were similar to the composition of OH-PCB metabolites (using the 3-8Cl PCB mixture

substrate) in our study. Applying dog microsome for *in vitro* PCB metabolism mostly generated 3-5Cl OH-PCBs in our results, while 6-8Cl OH-PCBs were reported to be the dominant OH-PCBs in the blood of dogs by Mizukawa et al., (2013). These observations suggested that phase II metabolism plays an important role in the excretion of 3-5Cl OH-PCB from the dog's body. In humans, the most common OH-PCB metabolites in serum samples were 4OH-CB107, 3′OH-PCB138, 4OH-PCB146, 3OH-PCB153 and 4OH-CB187 (Grimm et al., 2015; Park et al., 2009), whereas lower OH-PCBs have not been extensively characterized because of their rapid hydroxylation and consequent elimination (Grimm et al., 2015).

CYP3A contribution to PCB18 metabolism

The predicted pathways of CYP-mediated metabolism of PCBs in cats and dogs using *in vitro* metabolism and *in silico* analyses are shown in Fig. 7. CYP3A protein expression in microsomes tended to be lower in the PCB-exposed groups than in the control groups in dogs and cats. Our previous study also found that feline CYP3A12 and CYP3A131 mRNA expression in the liver of PCB exposure group was significantly downregulated compared to that in the control group (Khidkhan et al., 2019). Interestingly, the lower CYP3A protein expression could be associated with decreased 4′OH-CB18 formation by reducing CYP3A activity regarding the *in vitro* metabolism of PCB18 using microsomes of cats and dogs exposed to PCBs. Furthermore, *in silico* docking simulation confirmed that cat CYP3A and dog CYP3A/1A1 both have the potential to metabolize PCB18 to 4′OH-CB18. Since 4′OH-CB18 was the major metabolite for CYP-mediated PCB metabolism, the total levels of

metabolites were also consistent with the CYP3A protein expression in cat and dog microsomes. Our present results, in accordance with the previous *in vitro* metabolism study in Greenland sled dogs (*Canis familiaris*) suggested that CYP3A was responsible for the metabolism of PCB18 (Verreault et al., 2009). In addition, CYP3A possibly metabolized PCB136 in mice and rats (Kania-Korwel et al., 2008).

Low contributions of feline CYP1A1 and canine CYP1A/2B for the metabolism of PCBs

PCBs exposure significantly induced hepatic CYP1A1/1A2 protein expression in cats and CYP1A1/1A2 and CYP2B protein expressions in dogs. In cats, the expression of these protein was linked to the upregulation of CYP1A1 and CYP1A2 at the mRNA level in the liver of the PCB exposure group compared to that in the control (Khidkhan et al., 2019). *In vitro* PCB metabolism indicated that feline CYP1A might participate in the metabolism of PCB77 to 4'OH-CB79, PCB101 to 3OH-CB101, and PCB187 to 4OH-CB187. As for using dog microsome for *in vitro* metabolism, the results indicated that CYP1A and CYP2B might metabolize PCB28 to 4′OH-CB25 and 4′OH-CB35, PCB70 to 4OH-CB70, PCB77 to 4′OH-CB79, and PCB101 to 3OH-CB101 and 4OH-CB101. On the other hand, the *in silico* docking simulation analysis found that cat CYP3A132 and dog CYP1A1 and CYP3A12 are mainly responsible for those PCB substrates. Although these findings are difficult to interpret, one possible explanation is that the full saturations between PCB substrates and cat CYP3A or dog CYP1A/CYP3A resulted in the contributions of the cat CYP1A and dog CYP1A/2B in the metabolism of those PCBs observed *in vitro* results. In comparison to the level of major metabolites (4'OH-CB18), very small amounts of 4'OH-CB79, 3OH-CB101, and 4OH-

CB187 in cats, and 4'OH-CB25, 4'OH-CB35, 4OH-CB70, 4'OH-CB79, 3OH-CB101, and 4OH-CB101 in dogs were found within *in vitro* reactions; indicating cat CYP1A and dog CYP1A/2B contributed less to the metabolism of PCB77, PCB101 and PCB187, and PCB28, PCB70, PCB77, and PCB101, respectively.

CYP isoforms differ greatly in their biotransformation ability of PCB congeners (Hrycay and Bandiera, 2003); CYP1A and 2B are responsible for the metabolism of most PCBs and have been the main focus in several studies. *In vitro* metabolism studies using rat and human microsomes showed that CYP1A primarily metabolized PCB77, whereas CYP2B preferentially metabolized PCB47, PCB52, PCB54 and PCB153 (Ariyoshi et al., 1995; Hrycay and Bandiera, 2003; Richardson and Schlenk, 2011). Previous *in silico* analysis and *in vitro* study using Baikal seal CYPs and 62 PCBs suggest that CYP2A and CYP2B were extensive catalysts for PCBs, while CYP1A2 and CYP1B1 were poor catalysts (Yoo et al., 2015). In addition, CYP2A responsibly oxidized PCB101 and PCB52 in humans and hamsters, respectively (Koga et al., 1996; McGraw and Waller, 2006). However, this study was the first to suggest that feline CYP3A and canine CYP3A/1A1 could be major players in the metabolism of PCB congeners, such as PCB18, PCB28, PCB70, PCB77, PCB101, and PCB187, while the CYP1A1 in cats and CYP1A2/2B in dogs have fewer catalysts. Our study suggests that *in vitro* metabolism, together with *in silico* docking simulation is useful for predicting CYP-dependent metabolism of PCB congeners.

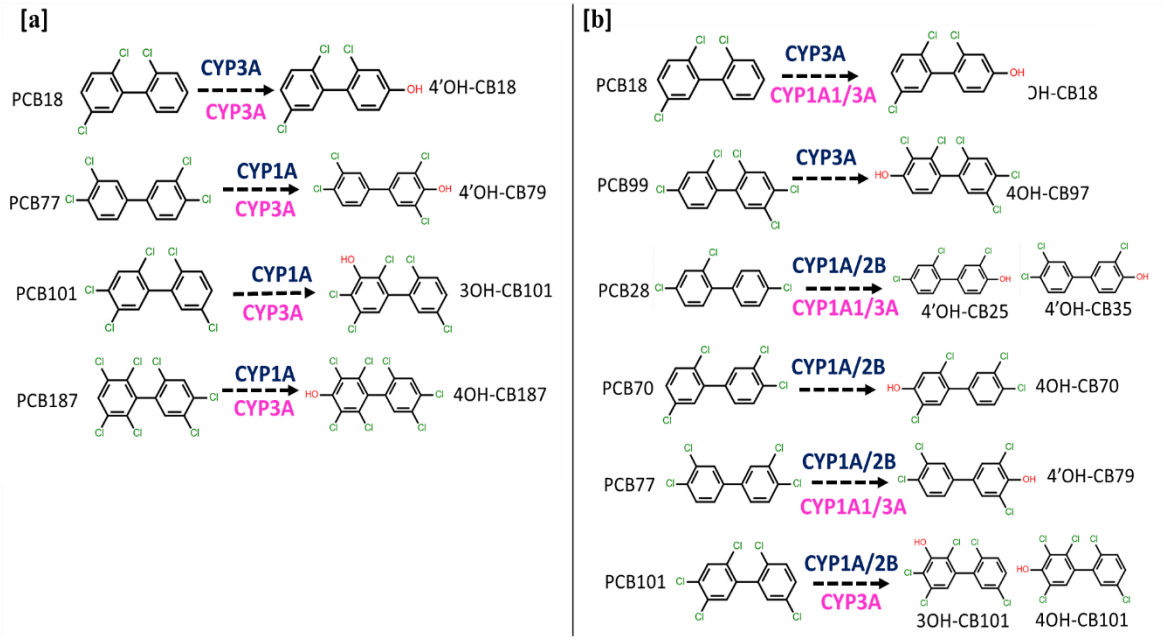

Fig. 7 Predicted pathways of CYPs metabolizing PCBs in cats [a] and dogs [b] after *in vitro* metabolism (blue character) and *in silico* analyses (pink character)

Results and Discussion: *Species variations of CYP-mediated metabolism of neonicotinoids among cat, dog, rat, and human*

Kinetic studies on imidacloprid

In vitro CYP activities for imidacloprid were measured and compared among rats, dogs, cats, and humans in this study. Although many metabolites of imidacloprid (including 4-hydroxy-imidacloprid (4OH-imidacloprid), 5-hydroxy-imidacloprid (5OH-imidacloprid), 5-methoxymethoxy-imidacloprid, 6-CNA, desnitro-dehydro-imidacloprid (dn-dh-imdacloprid), desnitro-imidacloprid (dn-imdacloprid) and imdacloprid olefin) have been reported (Dai et al., 2006; Ikenaka et al., 2018; Simon-Delso et al., 2015; Takahashi et al., 2019), 4OH-imidacloprid, 5OH-imidacloprid, dn-dh-imidacloprid, dn-imdacloprid, and

imidacloprid olefin were detected in all selected species. The kinetic parameters of 4OH-imidacloprid and 5OH-imidacloprid are presented in Table 4; other metabolites were detected in extremely low quantities and were only found in some reactions that involved a high substrate concentration; therefore, these could not be fit to the Michaelis-Menten plot. Our results suggested that *in vitro* reactions using liver microsomes in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent system may be suitable for hydroxylation of the imidazolidine ring at positions 4 or 5 yielding 4OH-imdacloprid and 5OH-imidacloprid. A recent study indicates 5OH-imidacloprid as the only metabolite of imidacloprid when rat and rainbow trout microsomes are used (Kolanczyk et al., 2020). Fig. 8 demonstrates the Michaelis-Menten plots for CYP activities when imidacloprid was used as a substrate and Table 4 shows the Michaelis-Menten parameters during formation of 4OH-imidacloprid and 5OH-imidacloprid in each species. Significant differences in the V_{max}/K_m values for 4OH-imidacloprid formation were found among species; the rate of oxidation of imidacloprid to 4OH-imidacloprid was found to be most rapid when rat liver microsomes were used, followed by that using human, dog, and cat microsomes (Fig. 8A and Table 4). The V_{max}/K_m values for 5OH-imidacloprid formation using rat and human microsomes were significantly higher than those using dog and cat microsomes (Fig. 8B and Table 4). A previous *in vitro* study (Kolanczyk et al., 2020) reports that the clearance of 5OH-imidacloprid in the SD rat (K_m = 158.7 µM, V_{max} = 38.4 pmol/min/mg) is much higher than that in rainbow trout (K_m = 79.2 µM, V_{max} = 0.75 pmol/min/mg). In humans, CYP2D6 and CYP3A4 are selective for the nitro-reduction of imidacloprid and formation of 5OH-imidacloprid, respectively (Casida, 2011; Schulz-Jander and Casida, 2002). Although human CYP3A4 is orthologous to rat

CYP3A9 and dog CYP3A12 (Court, 2013a; Martignoni et al., 2006; Xue et al., 2003), our results indicated that CYP3A in rats and humans may have a greater enzymatic affinity to imidacloprid as a substrate and could therefore more efficiently metabolize imidacloprid to 5OH-imidacloprid, as compared to CYP3A in dogs and cats.

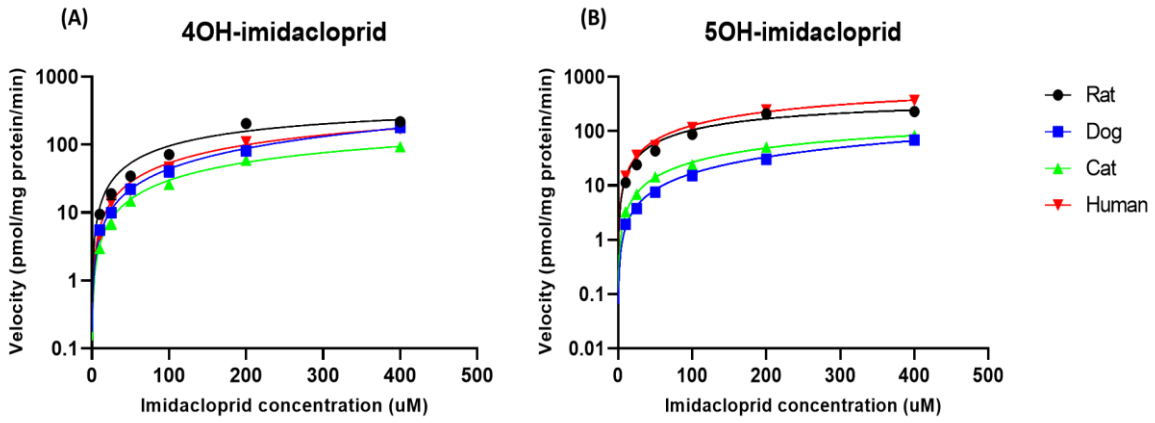

Fig. 8 Michaelis-Menten plots for CYP activity of imidacloprid substrates in SD rat (black circle), dog (blue square), cat (green triangle) and human (red triangle) liver microsomes

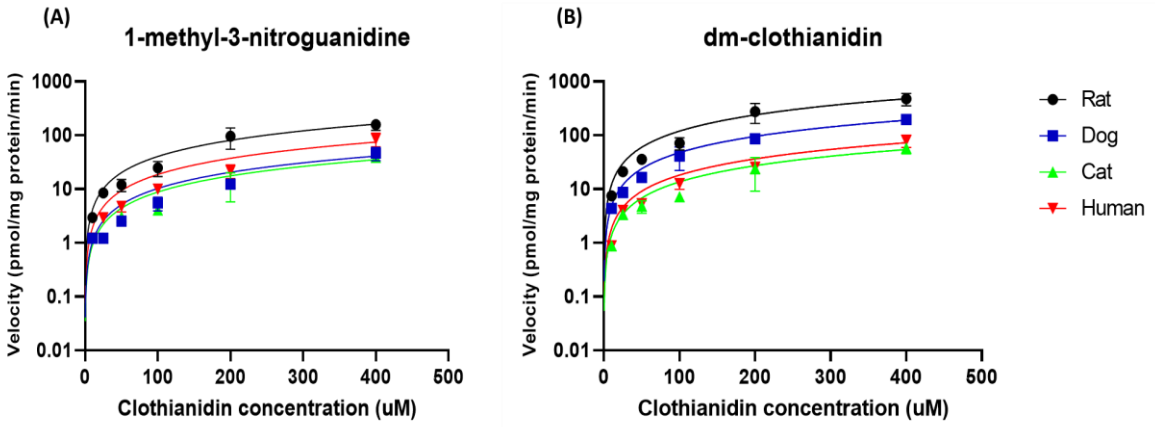

Fig. 9 Michaelis-Menten plots for CYP activity of clothianidin substrates in SD rat (black circle), dog (blue square), cat (green triangle), and human (red triangle) liver microsomes

Kinetic studies on clothianidin

The CYP metabolic assay for clothianidin indicated that 1-methyl-3-nitroguanidine and desmethyl-clothianidin (dm-clothianidin) were the major metabolites detected in all analyzed species. In mice, dm-clothianidin is the principal metabolite found in the brain and liver after exposure to clothianidin (Ford and Casida, 2006). As shown in Fig. 9 and Table 4, the clothianidin substrate was metabolized to 1-methyl-3-nitroguanidine formation (Fig. 9A) and dm-clothianidin formation (Fig. 9B) using rat microsomes significantly faster than it was when dog, human, and cat microsomes were used. A study of human CYP recombinant enzymes in NADPH-dependent reactions reveals that CYP3A4, CYP2C19, and CYP2A6 are responsible for converting clothianidin to dm-clothianidin (Ford and Casida, 2006; Shi et al., 2009). Among the four chosen species, our findings suggested that CYP3A4, CYP2C19, and CYP2A6 in rats may have higher clothianidin substrate-binding capacities and clothianidin demethylation ability than in other species. Interestingly, cats and humans showed no significant difference in clearances (V_{max}/K_m) of these metabolites.

Kinetic studies on acetamiprid

An *in vitro* acetamiprid metabolism assay using microsomes of the four species revealed the formation of several metabolites including 6-chloronicotinic acid (6-CNA), descyano-acetamiprid (dc-acetamiprid), desmethyl-acetamiprid (dm-acetamiprid), N-acetyl-acetamiprid, and N-acetyl-desmethyl-acetamiprid (N-acetyl-dm-acetamiprid). Among these metabolites (Table 4), the kinetics of N-acetyl-dm-acetamiprid were calculated for the microsomes obtained from humans and cats. 6-CNA (the final metabolites of acetamiprid)

was fit to the Michaelis-Menten curve in the reaction using rat microsomes, while it could not be detected in the reactions using dog microsomes. In addition, the levels of 6-CNA were not fit to the Michaelis-Menten equation in the reactions using the microsomes of cats and humans. 6-CNA is indicated as a common metabolite of chloropyridyl neonicotinoids, including imidacloprid and acetamiprid. It is excreted in human urine and has been proposed as an indicator in monitoring exposure to insecticides (Taira et al., 2011; Uroz et al., 2001). However, our findings indicated that 6-CNA may be not a suitable marker in dogs, cats, or humans exposed to acetamiprid and imidacloprid. Dc-acetamiprid was also found in all analyzed species; however, the detected levels were not fit to the Michaelis-Menten plot. Kolancyzk et al., (2020) report the detection of only a single metabolite, dm-acetamiprid, when acetamiprid is metabolized *in vitro* using rat and rainbow trout microsomes. While the kinetic parameters for microsomal demethylation of acetamiprid could not be quantified in rainbow trout, the kinetics of dm-acetamiprid in rats were determined (K_m = 70.9 µM, V_{max} = 10 pmol/min/mg) (Kolanczyk et al., 2020). In humans, dm-acetamiprid is the dominant metabolite of acetamiprid and is frequently detected in urine samples (Harada et al., 2016). As shown in Figs. 10A and 10B and in Table 4, the V_{max}/K_m values of dm-acetamiprid and N-acetyl-acetamiprid formation were significantly higher in humans than in other examined species. There is currently no published information regarding the specific CYP-mediated acetamiprid demethylation in humans or in other vertebrates. The residues of dm-acetamiprid in urine can be used as a marker to detect environmental exposure to acetamiprid in humans owing to the slow excretion of this metabolite (Harada et al., 2016; Marfo et al., 2015a; Marfo et al., 2015b). Therefore, the lower clearance (V_{max}/K_m) of acetamiprid and its metabolism to

dm-acetamiprid in rats, dogs, and cats, as compared to humans, suggested that dm-acetamiprid might serve as a marker to indicate environmental exposure to acetamiprid. However, interspecies differences in excretion of acetamiprid related to the enzymes involved in phase II metabolism should be considered.

Interspecies differences of metabolic capacities for neonicotinoid metabolism

In this study, the interspecies differences in neonicotinoid metabolites were analyzed, particularly those in acetamiprid metabolism. Results of the interspecies comparison of CYP activities for the *in vitro* metabolism of neonicotinoids, cats and dogs showed noticeable lower metabolic ability for imidacloprid, clothianidin and acetamiprid than in humans and rats. Phase I metabolism of neonicotinoids is largely dependent on CYP (Simon-Delso et al., 2015; Thompson et al., 2020); however, the CYP isoforms that were involved in the metabolism of neonicotinoids was unclear. Based on our results, it can be concluded that the interspecies differences in the metabolism of neonicotinoids might be due to the differences in expression levels of CYP isoforms in rats, dogs, cats, and humans. Previous studies report that glutathione conjugation, glycine conjugation, and glucuronidation in phase II metabolism are important in neonicotinoid clearance (Ford and Casida, 2006; Tomizawa and Casida, 2000). However, cats lack the metabolic capacity for glucuronidation (Court, 2013b). Although our results suggested approximately the same V_{max}/K_m values in cats and dogs in the metabolism of neonicotinoids, in reality, cats may display lesser metabolic capacity than dogs. Further studies should be directed at identifying the differences in hepatic metabolism of neonicotinoids in these species using recombinant CYP enzymes. Moreover, to clearly

define the species variations in toxicological risks involved in the exposure of neonicotinoids to household pets, the investigations on phase II metabolism, *in vivo* experiments, and biomonitoring exposures are needed.

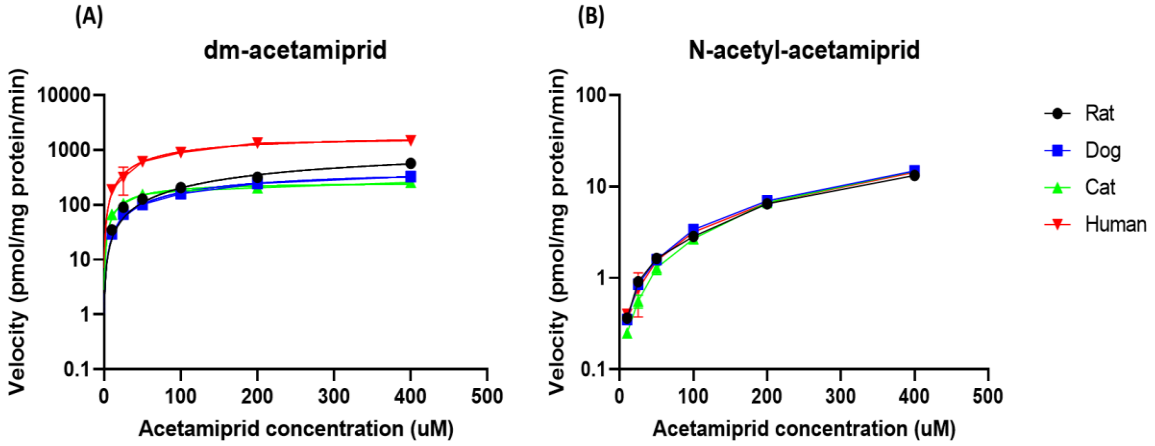

Fig. 10 Michaelis-Menten plots for CYP activity of acetamiprid substrates in SD rat (black circle), dog (blue square), cat (green triangle), and human (red triangle) liver microsomes

Table 4 Michaelis-Menten kinetic parameters (V_{max} (pmol/min/mg), K_m (µM), V_{max}/K_m, (µL/min/mg), mean ± SD) for CYP metabolism of neonicotinoids

Substrate	Metabolite	Parameter	Rat	Dog	Cat	Human
Imidacloprid	4OH-imidacloprid	V_{max}	454 ± 2	465 ± 606	336 ± 15	668 ± 68
		K_m	374 ± 2	1166 ± 1557	1025 ± 42	1142 ± 43
		V_{max}/K_m	1.2 ± 0.1 [A]	0.5 ± 0.1 [B]	0.3 ± 0.0 [B]	0.6 ± 0.0 [B]
	5OH-imidacloprid	V_{max}	440 ± 45	78 ± 53	283 ± 73	1042 ± 56
		K_m	320 ± 61	468 ± 358	958 ± 308	721 ± 17
		V_{max}/K_m	1.4 ± 0.1 [A]	0.2 ± 0.0 [B]	0.3 ± 0.0 [B]	1.5 ± 0.0 [A]
	5-methoxymethoxy-imidacloprid	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND
	6-CNA	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND
	dn-dh-imidacloprid	V_{max} K_m V_{max}/K_m	NF	NF	NF	NF
	dn-imidacloprid	V_{max} K_m V_{max}/K_m	NF	NF	NF	NF
	Imidacloprid-olefin	V_{max} K_m V_{max}/K_m	NF	NF	NF	NF
Clothianidin	1-methyl-3-nitroguanidine	V_{max}	26 ± 13	8 ± 6	8 ± 2	19 ± 15
		K_m	56 ± 31	113 ± 101	91 ± 28	141 ± 122
		V_{max}/K_m	0.5 ± 0.0 [A]	0.1 ± 0.0 [B]	0.1 ± 0.0 [B]	0.2 ± 0.0 [B]
	dm-clothianidin	V_{max}	105 ± 37	78 ± 1	23 ± 11	58. ± 13
		K_m	100 ± 30	189 ± 9	188 ± 107	391 ± 39
		V_{max}/K_m	1.1 ± 0.1 [A]	0.4 ± 0.0 [B]	0.1 ± 0.0 [C]	0.2 ± 0.0 [C]
	Clothianidin-urea	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND
	dm-clothianidin-urea	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND
	dm-dn-clothianidin	V_{max} K_m V_{max}/K_m	ND	ND	ND	ND

Table 4 Michaelis-Menten kinetic parameters (V_{max} (pmol/min/mg), K_m (μM), V_{max}/K_m, (μL/min/mg), mean ± SD) for CYP metabolism of neonicotinoids (continued)

Substrate	Metabolite	Parameter	Rat	Dog	Cat	Human
Acetamiprid	6-CNA	V_{max}	56 ± 12	ND	NF	NF
		K_m	310 ± 107			
		V_{max}/K_m	0.2 ± 0.0			
	dc-acetamiprid	V_{max}	NF	NF	NF	NF
		K_m				
		V_{max}/K_m				
	dm-acetamiprid	V_{max}	1344 ± 274	472 ± 20	260 ± 5	1940 ± 204
		K_m	565 ± 171	183 ± 5	36 ± 5	110 ± 38
		V_{max}/K_m	2.4 ± 0.2 [B]	2.5 ± 0.0 [B]	7.2 ± 1.2 [B]	17.7 ± 4.2 [A]
	dm-dc-acetamiprid	V_{max}	ND	ND	ND	ND
		K_m				
		V_{max}/K_m				
	N-acetyl-acetamiprid	V_{max}	11 ± 2	19 ± 16	5 ± 4	5 ± 0
		K_m	271 ± 5	527 ± 459	173 ± 158	96 ± 11
		V_{max}/K_m	0.039 ± 0.000 [AB]	0.037 ± 0.002 [B]	0.027 ± 0.001 [C]	0.047 ± 0.004 [A]
	N-acetyl-dm-acetamiprid	V_{max}	NF	NF	0.4 ± 0.3	0.5 ± 0.5
		K_m			244 ± 210	61 ± 152
		V_{max}/K_m			0.002 ± 0.001	0.008 ± 0.007

Different characters (A, B, and C) indicate statistically significant differences of V_{max}/K_m (Tukey's HSD test, $P < 0.05$), NF; Not fit to Michaelis-Menten plot, ND; Not detected

Conclusion

In conclusion, the 3-5Cl PCBs could have a higher binding affinity to CYPs than the 6-8 Cl PCBs in cats and dogs. According to *in vitro* and *in silico* results, most PCBs in dogs may be catalyzed by either CYP1A1, CYP3A, or both, while CYP1A2 and CYP2B11 contributed less in metabolizing PCBs. In cats, PCB congeners may be primarily catalyzed by CYP3A and are slightly contributed to by CYP1A1. Cat CYP3A and dog CYP3A/CYP1A1 might be responsible for metabolizing PCB18 to 4'OH-CB18, which is the dominant congener in both species. Moreover, this study constituted the first investigation of interspecies variations in CYP-mediated metabolism of neonicotinoids and the specific compositions of neonicotinoid metabolites detected using the hepatic microsomal fractions of rats, dogs, cats, and humans. The rate of formations of most neonicotinoid metabolites (including 4OH-imidacloprid, 5OH-imdacloprid, 1-methyl-3-nitroguanidine, and dm-clothianidin) was highest in rats, followed by that in humans, dogs and cats. The formation of dm-acetamiprid and N-acetyl-acetamiprid was highest in humans, followed by rats, dogs, and cats. These findings indicated that cats had a low capacity for PCB metabolism as compared to dogs, and for neonicotinoid metabolism as compared to rats and humans, which were confirmed using *in vitro* microsomal assay.

Chapter 4: Summary and future perspectives

Summary

These *in vivo* and *in vitro* studies aimed to elucidate the tissue distribution of CYP mRNA expression in cats; investigate the PCBs and BDE-209 that induced the expression of feline CYP isoforms; and compared feline CYP activity involved in PCB and neonicotinoid exposure to other species, including dogs, rats, and humans. Overall, CYP3A was the dominant subfamily in the liver. Some CYP isoforms (CYP1A1 and CYP2B11) were highly expressed in the extrahepatic tissues, whereas CYP2B, one of the most important isoforms for the metabolism of several substances, was not detected in the liver of cats. Chronic oral exposure (one year) to BDE-209 could not induce CYP1–CYP3 mRNA expression in the liver of cats, whereas the results after a single exposure to twelve PCB mixtures revealed that CYP1A1, CYP1A2 and CYP1B1 mRNA expression could be clearly induced by PCBs and may be strongly induced by CB77 in several tissues. In addition, these isozymes may play a significant role in PCB metabolism, especially 3–4 Cl-PCBs. However, the findings of *in vitro* PCB metabolism combined with *in silico* docking simulation indicated that feline CYP3A and canine CYP3A/1A1 mainly contribute to PCB metabolism, particularly PCB18 to 4′OH-CB18, which is the predominant metabolite in both cats and dogs. In addition, CYP1A1 in cats and CYP1A2/2B in dogs could be minor players for the metabolism of some PCB congeners (PCB28, PCB70, PCB77, PCB101, and PCB187) that led to their metabolites being formed at very small amounts. The 3-5Cl PCBs could have a higher binding affinity to CYPs than the 6-8Cl PCBs in cats and dogs. The significantly lower levels of OH-PCBs in reaction mixtures using the cat microsome suggested that cats have a lower capacity for PCB metabolism compared to dogs. The *in vitro* study on the interspecies differences in CYP

activities for neonicotinoid metabolism indicated that rats and humans, followed by dogs and cats had the highest clearance ability of most neonicotinoids, including 4OH-imidacloprid, 5OH-imidacloprid, 1-methyl-3-nitroguanidine, dm-clothianidin, dm-acetamiprid, and N-acetyl-acetamiprid. Our results suggested that cats and dogs have a low capacity for CYP-mediated neonicotinoid metabolism compared to rats and humans. In accordance with both *in vitro* CYP metabolic studies for PCBs and neonicotinoids, the OH-PCB levels and the kinetic parameters (V_{max}/K_m, V_{max} and K_m) for neonicotinoid metabolism implied that cats have low CYP ability for metabolizing these studied chemicals compared to other species, such as dogs, rats, and humans. In addition to CYP metabolism, phase II conjugation also plays an essential role in detoxification and excretion of chemicals. I suggested that the biotransformation of these studied compounds may occur less in cats compared to dogs or other species because cats not only have glucuronidation deficiency but also presented missing CYP2B in the liver as well as low CYP activity for these substances.

Recently, cats are popular pets that can be exposed to several environmental chemicals like humans, but the information on CYP isoforms involved in exposure and metabolism of numerous chemicals in cats is limited. Findings regarding detailed mechanisms of feline CYPs induced by environmental contaminants and further studies to define specific CYP isoforms for the metabolism of chemicals using recombinant feline CYP protein expression are particularly important. Moreover, the *in-silico* analyses, including docking simulation and molecular dynamics, will provide useful data for estimation pathways and possible toxicities of CYP-mediated chemical metabolism in cats.

References

Aggarwal, M., Battalora, M., Fisher, P., Hüser, A., Parr-Dobrzanski, R., Soufi, M., Mostert, V., Strupp, C., Whalley, P., Wiemann, C., Billington, R., 2014. Assessment of in vitro human dermal absorption studies on pesticides to determine default values, opportunities for read-across and influence of dilution on absorption. Regulatory toxicology and pharmacology : RTP 68, 412-423.

Ahlborg, U.G., Becking, G.C., Birnbaum, L.S., Brouwer, A., Derks, H., Feeley, M., Golor, G., Hanberg, A., Larsen, J.C., Liem, A.K.D., Safe, S.H., Schlatter, C., Waern, F., Younes, M., Yrjänheikki, E., 1994. Toxic equivalency factors for dioxin-like PCBs: Report on WHO-ECEH and IPCS consultation, December 1993. Chemosphere 28, 1049-1067.

Alexander, D.L., Eltom, S.E., Jefcoate, C.R., 1997. Ah receptor regulation of CYP1B1 expression in primary mouse embryo-derived cells. Cancer research 57, 4498-4506.

Ali, N., Malik, R.N., Mehdi, T., Eqani, S.A.M.A.S., Javeed, A., Neels, H., Covaci, A., 2013. Organohalogenated contaminants (OHCs) in the serum and hair of pet cats and dogs: Biosentinels of indoor pollution. Science of The Total Environment 449, 29-36.

Ali, U., Sweetman, A.J., Jones, K.C., Malik, R.N., 2018. Higher atmospheric levels and contribution of black carbon in soil-air partitioning of organochlorines in Lesser Himalaya. Chemosphere 191, 787-798.

Amano, I., Miyazaki, W., Iwasaki, T., Shimokawa, N., Koibuchi, N., 2010. The effect of hydroxylated polychlorinated biphenyl (OH-PCB) on thyroid hormone receptor

(TR)-mediated transcription through native-thyroid hormone response element (TRE). Industrial health 48, 115-118.

Anders, M.W., 1980. Metabolism of drugs by the kidney. Kidney International 18, 636-647.

Andersen, M.E., 1981. Saturable metabolism and its relationship to toxicity. Critical reviews in toxicology 9, 105-150.

Androutsopoulos, V.P., Tsatsakis, A.M., Spandidos, D.A., 2009. Cytochrome P450 CYP1A1: wider roles in cancer progression and prevention. BMC cancer 9, 187-187.

Ariyoshi, N., Oguri, K., Koga, N., Yoshimura, H., Funae, Y., 1995. Metabolism of highly persistent PCB congener, 2,4,5,2',4',5'-hexachlorobiphenyl, by human CYP2B6. Biochem Biophys Res Commun 212, 455-460.

Baillie, T.A., Rettie, A.E., 2011. Role of biotransformation in drug-induced toxicity: influence of intra- and inter-species differences in drug metabolism. Drug Metab Pharmacokinet 26, 15-29.

Barber, J.L., Walsh, M.J., Hewitt, R., Jones, K.C., Martin, F.L., 2006. Low-dose treatment with polybrominated diphenyl ethers (PBDEs) induce altered characteristics in MCF-7 cells. Mutagenesis 21, 351-360.

Bates, N., Rawson-Harris, P., Edwards, N., 2015. Common questions in veterinary toxicology. The Journal of small animal practice 56, 298-306.

Beischlag, T.V., Luis Morales, J., Hollingshead, B.D., Perdew, G.H., 2008. The aryl hydrocarbon receptor complex and the control of gene expression. Critical reviews in eukaryotic gene expression 18, 207-250.

Berheim, E.H., Jenks, J.A., Lundgren, J.G., Michel, E.S., Grove, D., Jensen, W.F., 2019. Effects of Neonicotinoid Insecticides on Physiology and Reproductive Characteristics of Captive Female and Fawn White-tailed Deer. Scientific Reports 9, 4534.

Beyer, A., Biziuk, M., 2009. Environmental fate and global distribution of polychlorinated biphenyls. Reviews of environmental contamination and toxicology 201, 137-158.

Bezirtzoglou, E.E.V., 2012. Intestinal cytochromes P450 regulating the intestinal microbiota and its probiotic profile. Microb Ecol Health Dis 23, 10.3402/mehd.v3423i3400.18370.

Bhalla, R., Tehrani, R., Van Aken, B., 2016. Toxicity of hydroxylated polychlorinated biphenyls (HO-PCBs) using the bioluminescent assay Microtox(®). Ecotoxicology 25, 1438-1444.

Björkholm, B., Bok, C.M., Lundin, A., Rafter, J., Hibberd, M.L., Pettersson, S., 2009. Intestinal Microbiota Regulate Xenobiotic Metabolism in the Liver. PLOS ONE 4, e6958.

Boersma, E.R., Lanting, C.I., 2000. Environmental exposure to polychlorinated biphenyls (PCBs) and dioxins. Consequences for longterm neurological and cognitive development of the child lactation. Advances in experimental medicine and biology 478, 271-287.

Bogaards, J.J., Bertrand, M., Jackson, P., Oudshoorn, M.J., Weaver, R.J., van Bladeren, P.J., Walther, B., 2000. Determining the best animal model for human cytochrome P450

activities: a comparison of mouse, rat, rabbit, dog, micropig, monkey and man. Xenobiotica; the fate of foreign compounds in biological systems 30, 1131-1152.

Boucher, B.A., Ennis, J.K., Tsirlin, D., Harris, S.A., 2018. A global database of polybrominated diphenyl ether flame retardant congeners in foods and supplements. Journal of Food Composition and Analysis 69, 171-188.

Braouezec, C., Enriquez, B., Blanchard, M., Chevreuil, M., Teil, M.J., 2016. Cat serum contamination by phthalates, PCBs, and PBDEs versus food and indoor air. Environmental science and pollution research international 23, 9574-9584.

Bui, P.H., Hankinson, O., 2009. Functional characterization of human cytochrome P450 2S1 using a synthetic gene-expressed protein in Escherichia coli. Molecular pharmacology 76, 1031-1043.

Casida, J.E., 2011. Neonicotinoid metabolism: compounds, substituents, pathways, enzymes, organisms, and relevance. J Agric Food Chem 59, 2923-2931.

Casida, J.E., 2018. Neonicotinoids and Other Insect Nicotinic Receptor Competitive Modulators: Progress and Prospects. Annual review of entomology 63, 125-144.

Chabot-Giguère, B., Letcher, R.J., Verreault, J., 2013. In vitro biotransformation of decabromodiphenyl ether (BDE-209) and Dechlorane Plus flame retardants: A case study of ring-billed gull breeding in a pollution hotspot in the St. Lawrence River, Canada. Environment International 55, 101-108.

Chandra Yadav, I., Devi, N.L., Li, J., Zhang, G., 2019. Examining the role of total organic carbon and black carbon in the fate of legacy persistent organic pollutants (POPs) in

indoor dust from Nepal: Implication on human health. Ecotoxicology and Environmental Safety 175, 225-235.

Chaudhary, K.R., Batchu, S.N., Seubert, J.M., 2009. Cytochrome P450 enzymes and the heart. IUBMB life 61, 954-960.

Cheung, Y.-L., Kerr, A.C., McFadyen, M.C.E., Melvin, W.T., Murray, G.I., 1999. Differential expression of CYP1A1, CYP1A2, CYP1B1 in human kidney tumours. Cancer Letters 139, 199-205.

Connor, K., Safe, S., Jefcoate, C.R., Larsen, M., 1995. Structure-dependent induction of CYP2B by polychlorinated biphenyl congeners in female Sprague-Dawley rats. Biochemical Pharmacology 50, 1913-1920.

Cortinovis, C., Caloni, F., 2016. Household Food Items Toxic to Dogs and Cats. Front Vet Sci 3, 26-26.

Costa, L.G., Giordano, G., 2007. Developmental neurotoxicity of polybrominated diphenyl ether (PBDE) flame retardants. Neurotoxicology 28, 1047-1067.

Costa, L.G., Giordano, G., 2011. Is decabromodiphenyl ether (BDE-209) a developmental neurotoxicant? Neurotoxicology 32, 9-24.

Court, M.H., 2013a. Canine cytochrome P450 (CYP) pharmacogenetics. The Veterinary clinics of North America. Small animal practice 43, 1027-1038.

Court, M.H., 2013b. Feline drug metabolism and disposition: pharmacokinetic evidence for species differences and molecular mechanisms. The Veterinary clinics of North America. Small animal practice 43, 1039-1054.

Court, M.H., Greenblatt, D.J., 2000. Molecular genetic basis for deficient acetaminophen glucuronidation by cats: UGT1A6 is a pseudogene, and evidence for reduced diversity of expressed hepatic UGT1A isoforms. Pharmacogenetics 10, 355-369.

Dai, Y.J., Yuan, S., Ge, F., Chen, T., Xu, S.C., Ni, J.P., 2006. Microbial hydroxylation of imidacloprid for the synthesis of highly insecticidal olefin imidacloprid. Applied microbiology and biotechnology 71, 927-934.

DellaValle, C.T., Wheeler, D.C., Deziel, N.C., De Roos, A.J., Cerhan, J.R., Cozen, W., Severson, R.K., Flory, A.R., Locke, S.J., Colt, J.S., Hartge, P., Ward, M.H., 2013. Environmental determinants of polychlorinated biphenyl concentrations in residential carpet dust. Environmental science & technology 47, 10405-10414.

Dhers, L., Ducassou, L., Boucher, J.L., Mansuy, D., 2017. Cytochrome P450 2U1, a very peculiar member of the human P450s family. Cellular and molecular life sciences : CMLS 74, 1859-1869.

Ding, X., Staudinger, J.L., 2005. Repression of PXR-mediated induction of hepatic CYP3A gene expression by protein kinase C. Biochem Pharmacol 69, 867-873.

Dirtu, A.C., Niessen, S.J., Jorens, P.G., Covaci, A., 2013. Organohalogenated contaminants in domestic cats' plasma in relation to spontaneous acromegaly and type 2 diabetes mellitus: a clue for endocrine disruption in humans? Environ Int 57-58, 60-67.

Donat-Vargas, C., Bellavia, A., Berglund, M., Glynn, A., Wolk, A., Akesson, A., 2019. Cardiovascular and cancer mortality in relation to dietary polychlorinated biphenyls and marine polyunsaturated fatty acids: a nutritional-toxicological aspect of fish consumption. Journal of internal medicine.

Dye, J.A., Venier, M., Zhu, L., Ward, C.R., Hites, R.A., Birnbaum, L.S., 2007. Elevated PBDE Levels in Pet Cats: Sentinels for Humans? Environmental Science & Technology 41, 6350-6356.

Eagling, V.A., Tjia, J.F., Back, D.J., 1998. Differential selectivity of cytochrome P450 inhibitors against probe substrates in human and rat liver microsomes. British journal of clinical pharmacology 45, 107-114.

Eguchi, A., Nomiyama, K., Ochiai, M., Mizukawa, H., Nagano, Y., Nakagawa, K., Tanaka, K., Miyagawa, H., Tanabe, S., 2014. Simultaneous detection of multiple hydroxylated polychlorinated biphenyls from a complex tissue matrix using gas chromatography/isotope dilution mass spectrometry. Talanta 118, 253-261.

El-Sherbeni, A.A., El-Kadi, A.O., 2014. Characterization of arachidonic acid metabolism by rat cytochrome P450 enzymes: the involvement of CYP1As. Drug metabolism and disposition: the biological fate of chemicals 42, 1498-1507.

Elbekai, R.H., El-Kadi, A.O.S., 2006. Cytochrome P450 enzymes: Central players in cardiovascular health and disease. Pharmacology & Therapeutics 112, 564-587.

Erratico, C.A., Moffatt, S.C., Bandiera, S.M., 2011. Comparative oxidative metabolism of BDE-47 and BDE-99 by rat hepatic microsomes. Toxicological sciences : an official journal of the Society of Toxicology 123, 37-47.

Erratico, C.A., Szeitz, A., Bandiera, S.M., 2012. Oxidative metabolism of BDE-99 by human liver microsomes: predominant role of CYP2B6. Toxicological sciences : an official journal of the Society of Toxicology 129, 280-292.

Evans, W.E., Relling, M.V., 1999. Pharmacogenomics: Translating Functional Genomics into Rational Therapeutics. Science 286, 487.

Fang, C., Zhang, C., Xia, G., Yang, W., 2002. Damaging effects of polychlorinated biphenyls on chicken primordial germ cells. Reproduction, fertility, and development 14, 177-183.

Feo, M.L., Gross, M.S., McGarrigle, B.P., Eljarrat, E., Barcelo, D., Aga, D.S., Olson, J.R., 2013. Biotransformation of BDE-47 to potentially toxic metabolites is predominantly mediated by human CYP2B6. Environ Health Perspect 121, 440-446.

Ferguson, C.S., Tyndale, R.F., 2011. Cytochrome P450 enzymes in the brain: emerging evidence of biological significance. Trends in pharmacological sciences 32, 708-714.

Ford, K.A., Casida, J.E., 2006. Unique and common metabolites of thiamethoxam, clothianidin, and dinotefuran in mice. Chemical research in toxicology 19, 1549-1556.

Gahrs, M., Roos, R., Andersson, P.L., Schrenk, D., 2013. Role of the nuclear xenobiotic receptors CAR and PXR in induction of cytochromes P450 by non-dioxinlike polychlorinated biphenyls in cultured rat hepatocytes. Toxicol Appl Pharmacol 272, 77-85.

Ghanim, M., Ishaaya, I., 2011. Insecticides with Novel Modes of Action Mechanism and Resistance Management, 385-407.

Ghosh, C., Hossain, M., Solanki, J., Dadas, A., Marchi, N., Janigro, D., 2016. Pathophysiological implications of neurovascular P450 in brain disorders. Drug discovery today 21, 1609-1619.

Gibbons, D., Morrissey, C., Mineau, P., 2015. A review of the direct and indirect effects of neonicotinoids and fipronil on vertebrate wildlife. Environmental science and pollution research international 22, 103-118.

Girolami, F., Spalenza, V., Manzini, L., Carletti, M., Nebbia, C., 2015. Constitutive expression of the AHR signaling pathway in a bovine mammary epithelial cell line and modulation by dioxin-like PCB and other AHR ligands. Toxicology Letters 232, 98-105.

González-Gómez, X., Cambeiro-Pérez, N., Martínez-Carballo, E., Simal-Gándara, J., 2018. Screening of organic pollutants in pet hair samples and the significance of environmental factors. Science of The Total Environment 625, 311-319.

Gonzalez Sagrario Mde, L., Miglioranza, K.S., Aizpun de Moreno, J.E., Moreno, V.J., Escalante, A.H., 2002. Polychlorinated biphenyls in different trophic levels from a shallow lake in Argentina. Chemosphere 48, 1113-1122.

Goto, J., Kataoka, R., Muta, H., Hirayama, N., 2008. ASEDock-Docking Based on Alpha Spheres and Excluded Volumes. Journal of Chemical Information and Modeling 48, 583-590.

Graham, M.J., Lake, B.G., 2008. Induction of drug metabolism: species differences and toxicological relevance. Toxicology 254, 184-191.

Grimm, F.A., Hu, D., Kania-Korwel, I., Lehmler, H.J., Ludewig, G., Hornbuckle, K.C., Duffel, M.W., Bergman, A., Robertson, L.W., 2015. Metabolism and metabolites of polychlorinated biphenyls. Critical reviews in toxicology 45, 245-272.

Guo, W., Park, J.S., Wang, Y., Gardner, S., Baek, C., Petreas, M., Hooper, K., 2012. High polybrominated diphenyl ether levels in California house cats: house dust a primary source? Environmental toxicology and chemistry 31, 301-306.

Han, Z., Li, Y., Zhang, S., Song, N., Xu, H., Dang, Y., Liu, C., Giesy, J.P., Yu, H., 2017. Prenatal transfer of decabromodiphenyl ether (BDE-209) results in disruption of the thyroid system and developmental toxicity in zebrafish offspring. Aquatic toxicology (Amsterdam, Netherlands) 190, 46-52.

Handa, K., Nakagome, I., Yamaotsu, N., Gouda, H., Hirono, S., 2013. In silico study on the inhibitory interaction of drugs with wild-type CYP2D6.1 and the natural variant CYP2D6.17. Drug Metab Pharmacokinet advpub.

Harada, K.H., Tanaka, K., Sakamoto, H., Imanaka, M., Niisoe, T., Hitomi, T., Kobayashi, H., Okuda, H., Inoue, S., Kusakawa, K., Oshima, M., Watanabe, K., Yasojima, M., Takasuga, T., Koizumi, A., 2016. Biological Monitoring of Human Exposure to Neonicotinoids Using Urine Samples, and Neonicotinoid Excretion Kinetics. PloS one 11, e0146335-e0146335.

Hay Kraus, B.L., Greenblatt, D.J., Venkatakrishnan, K., Court, M.H., 2000. Evidence for propofol hydroxylation by cytochrome P4502B11 in canine liver microsomes: breed and gender differences. Xenobiotica; the fate of foreign compounds in biological systems 30, 575-588.

Hedlund, E., Gustafsson, J.A., Warner, M., 2001. Cytochrome P450 in the brain; a review. Current drug metabolism 2, 245-263.

Hedrich, W.D., Hassan, H.E., Wang, H., 2016. Insights into CYP2B6-mediated drug–drug interactions. Acta Pharmaceutica Sinica. B 6, 413-425.

Henríquez-Hernández, L.A., Carretón, E., Camacho, M., Montoya-Alonso, J.A., Boada, L.D., Bernal Martín, V., Falcón Cordón, Y., Falcón Cordón, S., Zumbado, M., Luzardo, O.P., 2017. Potential Role of Pet Cats As a Sentinel Species for Human Exposure to Flame Retardants. Front Vet Sci 4, 79.

Hirakawa, S., Miyawaki, T., Hori, T., Kajiwara, J., Katsuki, S., Hirano, M., Yoshinouchi, Y., Iwata, H., Mitoma, C., Furue, M., 2018. Accumulation properties of polychlorinated biphenyl congeners in Yusho patients and prediction of their cytochrome P450-dependent metabolism by in silico analysis. Environmental science and pollution research international 25, 16455-16463.

Hoffman, S.M., Nelson, D.R., Keeney, D.S., 2001. Organization, structure and evolution of the CYP2 gene cluster on human chromosome 19. Pharmacogenetics 11, 687-698.

Honda, K., Komatsu, T., Koyama, F., Kubota, A., Kawakami, K., Asakura, H., Uno, Y., Kitazawa, T., Hiraga, T., Teraoka, H., 2011. Expression of two novel cytochrome P450 3A131 and 3A132 in liver and small intestine of domestic cats. The Journal of veterinary medical science 73, 1489-1492.

Hrycay, E.G., Bandiera, S.M., 2003. Spectral interactions of tetrachlorobiphenyls with hepatic microsomal cytochrome P450 enzymes. Chemico-Biological Interactions 146, 285-296.

Hukkanen, J., Pelkonen, O., Hakkola, J., Raunio, H., 2002. Expression and regulation of xenobiotic-metabolizing cytochrome P450 (CYP) enzymes in human lung. Critical reviews in toxicology 32, 391-411.

Ichikawa, G., Kuribayashi, R., Ikenaka, Y., Ichise, T., Nakayama, S.M.M., Ishizuka, M., Taira, K., Fujioka, K., Sairenchi, T., Kobashi, G., Bonmatin, J.M., Yoshihara, S., 2019. LC-ESI/MS/MS analysis of neonicotinoids in urine of very low birth weight infants at birth. PLoS One 14, e0219208.

Ikenaka, Y., Fujioka, K., Kawakami, T., Ichise, T., Bortey-Sam, N., Nakayama, S.M.M., Mizukawa, H., Taira, K., Takahashi, K., Kato, K., Arizono, K., Ishizuka, M., 2018. Contamination by neonicotinoid insecticides and their metabolites in Sri Lankan black tea leaves and Japanese green tea leaves. Toxicology Reports 5, 744-749.

Ikenaka, Y., Miyabara, Y., Ichise, T., Nakayama, S., Nimako, C., Ishizuka, M., Tohyama, C., 2019. Exposures of children to neonicotinoids in pine wilt disease control areas. Environmental toxicology and chemistry 38, 71-79.

Ishii, M., Toda, T., Ikarashi, N., Ochiai, W., Sugiyama, K., 2012. [Effects of intestinal flora on the expression of cytochrome P450 3A in the liver]. Yakugaku zasshi : Journal of the Pharmaceutical Society of Japan 132, 301-310.

Jover, R., Bort, R., Gomez-Lechon, M.J., Castell, J.V., 2002. Down-regulation of human CYP3A4 by the inflammatory signal interleukin-6: molecular mechanism and transcription factors involved. FASEB journal : official publication of the Federation of American Societies for Experimental Biology 16, 1799-1801.

Kakehi, M., Ikenaka, Y., Nakayama, S.M., Kawai, Y.K., Watanabe, K.P., Mizukawa, H., Nomiyama, K., Tanabe, S., Ishizuka, M., 2015. Uridine Diphosphate-Glucuronosyltransferase (UGT) Xenobiotic Metabolizing Activity and Genetic Evolution in Pinniped Species. Toxicological sciences : an official journal of the Society of Toxicology 147, 360-369.

Kamata, S., Fujino, N., Yamada, M., Grime, K., Suzuki, S., Ota, C., Tando, Y., Okada, Y., Sakurada, A., Noda, M., Matsuda, Y., Sugiura, H., Ichinose, M., 2018. Expression of cytochrome P450 mRNAs in Type II alveolar cells from subjects with chronic obstructive pulmonary disease. Pharmacology research & perspectives 6, e00405-e00405.

Kania-Korwel, I., Hrycay, E.G., Bandiera, S.M., Lehmler, H.J., 2008. 2,2',3,3',6,6'-Hexachlorobiphenyl (PCB 136) atropisomers interact enantioselectively with hepatic microsomal cytochrome P450 enzymes. Chemical research in toxicology 21, 1295-1303.

Kaw, H.Y., Kannan, N., 2017. A Review on Polychlorinated Biphenyls (PCBs) and Polybrominated Diphenyl Ethers (PBDEs) in South Asia with a Focus on Malaysia. Reviews of environmental contamination and toxicology 242, 153-181.

Kawano, M., Hasegawa, J., Enomoto, T., Onishi, H., Nishio, Y., Matsuda, M., Wakimoto, T., 2005. Hydroxylated polychlorinated biphenyls (OH-PCBs): recent advances in wildlife contamination study. Environmental sciences : an international journal of environmental physiology and toxicology 12, 315-324.

Khidkhan, K., Mizukawa, H., Ikenaka, Y., Nakayama, S.M.M., Nomiyama, K., Yokoyama, N., Ichii, O., Darwish, W.S., Takiguchi, M., Tanabe, S., Ishizuka, M., 2019. Tissue distribution and characterization of feline cytochrome P450 genes related to polychlorinated biphenyl exposure. Comparative biochemistry and physiology. Toxicology & pharmacology : CBP 226, 108613.

Klincic, D., Dvorscak, M., Jagic, K., Mendas, G., Herceg Romanic, S., 2020. Levels and distribution of polybrominated diphenyl ethers in humans and environmental compartments: a comprehensive review of the last five years of research. Environmental science and pollution research international.

Knights, K.M., Rowland, A., Miners, J.O., 2013. Renal drug metabolism in humans: the potential for drug-endobiotic interactions involving cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT). British journal of clinical pharmacology 76, 587-602.

Kodavanti, P.R.S., 2017. Polychlorinated Biphenyls (PCBs)☆, Reference Module in Neuroscience and Biobehavioral Psychology. Elsevier.

Kodavanti, P.R.S., Loganathan, B.G., 2017. Organohalogen Pollutants and Human Health, in: S.R. Quah (Ed.), International Encyclopedia of Public Health (Second Edition). Academic Press, Oxford, 359-366.

Kodavanti, P.R.S., Senthil Kumar, K., Loganathan, B.G., 2008. Organohalogen Pollutants and Human Health, in: H.K. Heggenhougen (Ed.), International Encyclopedia of Public Health. Academic Press, Oxford, 686-693.

Koenig, S., Fernández, P., Solé, M., 2012. Differences in cytochrome P450 enzyme activities between fish and crustacea: Relationship with the bioaccumulation patterns of polychlorobiphenyls (PCBs). Aquatic Toxicology 108, 11-17.

Koga, N., Kikuichi, N., Kanamaru, T., Ariyoshi, N., Oguri, K., Yoshimura, H., 1996. Hamster Liver Cytochrome P450 (CYP2A8) as a 4-Hydroxylase for 2,5,2′,5′-Tetrachlorobiphenyl. Biochemical and Biophysical Research Communications 225, 685-688.

Kolanczyk, R.C., Tapper, M.A., Sheedy, B.R., Serrano, J.A., 2020. In vitro metabolism of imidacloprid and acetamiprid in rainbow trout and rat. Xenobiotica; the fate of foreign compounds in biological systems 50, 805-814.

Komatsu, T., Honda, K., Kubota, A., Kitazawa, T., Hiraga, T., Teraoka, H., 2010. Molecular cloning and expression of cytochrome P450 2D6 in the livers of domestic cats. The Journal of veterinary medical science 72, 1633-1636.

Korytko, P.J., Casey, A.C., Bush, B., Quimby, F.W., 1999. Induction of hepatic cytochromes P450 in dogs exposed to a chronic low dose of polychlorinated biphenyls. Toxicological sciences : an official journal of the Society of Toxicology 47, 52-61.

Kovalkovičová, N., Sutiaková, I., Pistl, J., Sutiak, V., 2009. Some food toxic for pets. Interdiscip Toxicol 2, 169-176.

Laemmli, U.K., 1970. Cleavage of Structural Proteins during the Assembly of the Head of Bacteriophage T4. Nature 227, 680-685.

Larigot, L., Juricek, L., Dairou, J., Coumoul, X., 2018. AhR signaling pathways and regulatory functions. Biochimie open 7, 1-9.

Larsen, M.C., Angus, W.G., Brake, P.B., Eltom, S.E., Sukow, K.A., Jefcoate, C.R., 1998. Characterization of CYP1B1 and CYP1A1 expression in human mammary epithelial cells: role of the aryl hydrocarbon receptor in polycyclic aromatic hydrocarbon metabolism. Cancer research 58, 2366-2374.

Lascelles, B.D., Court, M.H., Hardie, E.M., Robertson, S.A., 2007. Nonsteroidal anti-inflammatory drugs in cats: a review. Veterinary anaesthesia and analgesia 34, 228-250.

Lau, G., Walter, K., Kass, P., Puschner, B., 2017. Comparison of polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs) in the serum of hypothyroxinemic and euthyroid dogs. PeerJ 5, e3780.

Lautier, T., Urban, P., Loeper, J., Jezequel, L., Pompon, D., Truan, G., 2016. Ordered chimerogenesis applied to CYP2B P450 enzymes. Biochimica et Biophysica Acta (BBA) - General Subjects 1860, 1395-1403.

Lee, E., Kim, T.H., Choi, J.S., Nabanata, P., Kim, N.Y., Ahn, M.Y., Jung, K.K., Kang, I.H., Kim, T.S., Kwack, S.J., Park, K.L., Kim, S.H., Kang, T.S., Lee, J., Lee, B.M., Kim, H.S., 2010. Evaluation of liver and thyroid toxicity in Sprague-Dawley rats after exposure to polybrominated diphenyl ether BDE-209. The Journal of toxicological sciences 35, 535-545.

Lewis, D.F.V., Lake, B.G., Bird, M.G., Loizou, G.D., Dickins, M., Goldfarb, P.S., 2003. Homology modelling of human CYP2E1 based on the CYP2C5 crystal structure: investigation of enzyme–substrate and enzyme–inhibitor interactions. Toxicology in Vitro 17, 93-105.

Li, C.X., Li, M., Feng, X.L., Cao, P., Wang, X.D., Liu, S., Xu, H.B., 2012. [Study on dermal absorption of Imidacloprid in vitro]. Zhonghua lao dong wei sheng zhi ye bing za zhi = Zhonghua laodong weisheng zhiyebing zazhi = Chinese journal of industrial hygiene and occupational diseases 30, 604-607.

Li, J., Zhang, Y., Du, Z., Peng, J., Mao, L., Gao, S., 2016. Biotransformation of OH-PBDEs by pig liver microsomes: Investigating kinetics, identifying metabolites, and examining the role of different CYP isoforms. Chemosphere 148, 354-360.

Lin, P.C., Lin, H.J., Liao, Y.Y., Guo, H.R., Chen, K.T., 2013. Acute poisoning with neonicotinoid insecticides: a case report and literature review. Basic & clinical pharmacology & toxicology 112, 282-286.

Livak, K.J., Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods (San Diego, Calif.) 25, 402-408.

Lohr, J.W., Willsky, G.R., Acara, M.A., 1998. Renal Drug Metabolism. Pharmacological Reviews 50, 107.

Ma, Y., Li, P., Jin, J., Wang, Y., Wang, Q., 2017. Current halogenated flame retardant concentrations in serum from residents of Shandong Province, China, and temporal changes in the concentrations. Environmental Research 155, 116-122.

Machala, M., Neča, J., Drábek, P., Ulrich, R., Šabatová, V., Nezveda, K., Raszyk, J., Gajdušková, V., 1998. Effects of chronic exposure to PCBs on cytochrome P450 systems and steroidogenesis in liver and testis of bulls (Bos taurus). Comparative

Biochemistry and Physiology Part A: Molecular & Integrative Physiology 120, 65-70.

Makey, C.M., McClean, M.D., Braverman, L.E., Pearce, E.N., He, X.M., Sjödin, A., Weinberg, J.M., Webster, T.F., 2016. Polybrominated Diphenyl Ether Exposure and Thyroid Function Tests in North American Adults. Environ Health Perspect 124, 420-425.

Marfo, J.T., Fujioka, K., Ikenaka, Y., Nakayama, S.M., Mizukawa, H., Aoyama, Y., Ishizuka, M., Taira, K., 2015a. Relationship between Urinary N-Desmethyl-Acetamiprid and Typical Symptoms including Neurological Findings: A Prevalence Case-Control Study. PLoS One 10, e0142172.

Marfo, J.T., Fujioka, K., Ikenaka, Y., Nakayama, S.M.M., Mizukawa, H., Aoyama, Y., Ishizuka, M., Taira, K., 2015b. Relationship between Urinary N-Desmethyl-Acetamiprid and Typical Symptoms including Neurological Findings: A Prevalence Case-Control Study. PLOS ONE 10, e0142172.

Martignoni, M., Groothuis, G.M., de Kanter, R., 2006. Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. Expert opinion on drug metabolism & toxicology 2, 875-894.

Martinez, M.N., Antonovic, L., Court, M., Dacasto, M., Fink-Gremmels, J., Kukanich, B., Locuson, C., Mealey, K., Myers, M.J., Trepanier, L., 2013. Challenges in exploring the cytochrome P450 system as a source of variation in canine drug pharmacokinetics. Drug metabolism reviews 45, 218-230.

McDonald, T.A., 2005. Polybrominated diphenylether levels among United States residents: daily intake and risk of harm to the developing brain and reproductive organs. Integrated environmental assessment and management 1, 343-354.

McGraw, J.E., Waller, D.P., 2006. Specific human CYP 450 isoform metabolism of a pentachlorobiphenyl (PCB-IUPAC# 101). Biochemical and Biophysical Research Communications 344, 129-133.

Mehlhorn, H., Hansen, O., Mencke, N., 2001. Comparative study on the effects of three insecticides (fipronil, imidacloprid, selamectin) on developmental stages of the cat flea (Ctenocephalides felis Bouché 1835): a light and electron microscopic analysis of in vivo and in vitro experiments. Parasitology research 87, 198-207.

Mencke, N., Jeschke, P., 2002. Therapy and Prevention of Parasitic Insects in Veterinary Medicine using Imidacloprid. Current topics in medicinal chemistry 2, 701-715.

Meng, F.-d., Ma, P., Sui, C.-g., Tian, X., Jiang, Y.-h., 2015. Association between cytochrome P450 1A1 (CYP1A1) gene polymorphisms and the risk of renal cell carcinoma: a meta-analysis. Scientific Reports 5, 8108.

Mensching, D.A., Slater, M., Scott, J.W., Ferguson, D.C., Beasley, V.R., 2012. The feline thyroid gland: a model for endocrine disruption by polybrominated diphenyl ethers (PBDEs)? Journal of toxicology and environmental health. Part A 75, 201-212.

Meyer, R.P., Podvinec, M., Meyer, U.A., 2002. Cytochrome P450 CYP1A1 accumulates in the cytosol of kidney and brain and is activated by heme. Molecular pharmacology 62, 1061-1067.

Mills, R.A., Millis, C.D., Dannan, G.A., Guengerich, F.P., Aust, S.D., 1985. Studies on the structure-activity relationships for the metabolism of polybrominated biphenyls by rat liver microsomes. Toxicol Appl Pharmacol 78, 96-104.

Miyazaki, W., Iwasaki, T., Takeshita, A., Kuroda, Y., Koibuchi, N., 2004. Polychlorinated biphenyls suppress thyroid hormone receptor-mediated transcription through a novel mechanism. The Journal of biological chemistry 279, 18195-18202.

Mizukawa, H., Nomiyama, K., Nakatsu, S., Yachimori, S., Hayashi, T., Tashiro, Y., Nagano, Y., Tanabe, S., 2013. Species-specific differences in the accumulation features of organohalogen contaminants and their metabolites in the blood of Japanese terrestrial mammals. Environmental pollution (Barking, Essex : 1987) 174, 28-37.

Morrissey, C.A., Mineau, P., Devries, J.H., Sanchez-Bayo, F., Liess, M., Cavallaro, M.C., Liber, K., 2015. Neonicotinoid contamination of global surface waters and associated risk to aquatic invertebrates: a review. Environ Int 74, 291-303.

Nair, P., McKinnon, R., Miners, J., 2016. Cytochrome P450 structure–function: insights from molecular dynamics simulations. Drug metabolism reviews 48, 1-19.

Nebert, D.W., Dalton, T.P., 2006. The role of cytochrome P450 enzymes in endogenous signalling pathways and environmental carcinogenesis. Nature reviews. Cancer 6, 947-960.

Nomiyama, K., Murata, S., Kunisue, T., Yamada, T.K., Mizukawa, H., Takahashi, S., Tanabe, S., 2010. Polychlorinated biphenyls and their hydroxylated metabolites (OH-PCBs) in the blood of toothed and baleen whales stranded along Japanese coastal waters. Environ Sci Technol 44, 3732-3738.

Nomiyama, K., Takaguchi, K., Mizukawa, H., Nagano, Y., Oshihoi, T., Nakatsu, S., Kunisue, T., Tanabe, S., 2017. Species- and Tissue-Specific Profiles of Polybrominated Diphenyl Ethers and Their Hydroxylated and Methoxylated Derivatives in Cats and Dogs. Environ Sci Technol 51, 5811-5819.

Norrgran, J., Jones, B., Bignert, A., Athanassiadis, I., Bergman, A., 2015. Higher PBDE serum concentrations may be associated with feline hyperthyroidism in Swedish cats. Environ Sci Technol 49, 5107-5114.

Ogu, C.C., Maxa, J.L., 2000. Drug interactions due to cytochrome P450. Proceedings (Baylor University. Medical Center) 13, 421-423.

Ohtsuki, S., Schaefer, O., Kawakami, H., Inoue, T., Liehner, S., Saito, A., Ishiguro, N., Kishimoto, W., Ludwig-Schwellinger, E., Ebner, T., Terasaki, T., 2012. Simultaneous Absolute Protein Quantification of Transporters, Cytochromes P450, and UDP-Glucuronosyltransferases as a Novel Approach for the Characterization of Individual Human Liver: Comparison with mRNA Levels and Activities. Drug Metabolism and Disposition 40, 83-92.

Okamatsu, G., Kawakami, K., Komatsu, T., Kitazawa, T., Uno, Y., Teraoka, H., 2017a. Functional expression and comparative characterization of four feline P450 cytochromes using fluorescent substrates. Xenobiotica; the fate of foreign compounds in biological systems 47, 951-961.

Okamatsu, G., Komatsu, T., Ono, Y., Inoue, H., Uchide, T., Onaga, T., Endoh, D., Kitazawa, T., Hiraga, T., Uno, Y., Teraoka, H., 2017b. Characterization of feline cytochrome

P450 2B6. Xenobiotica; the fate of foreign compounds in biological systems 47, 93-102.

Omura, T., Sato, R., 1964. THE CARBON MONOXIDE-BINDING PIGMENT OF LIVER MICROSOMES. I. EVIDENCE FOR ITS HEMOPROTEIN NATURE. The Journal of biological chemistry 239, 2370-2378.

Ono, Y., Sugiyama, S., Matsushita, M., Kitazawa, T., Amano, T., Uno, Y., Ikushiro, S., Teraoka, H., 2019. Limited expression of functional cytochrome p450 2c subtypes in the liver and small intestine of domestic cats. Xenobiotica; the fate of foreign compounds in biological systems 49, 627-635.

Otyepka, M., Berka, K., Anzenbacher, P., 2011. Is There a Relationship Between the Substrate Preferences and Structural Flexibility of Cytochromes P450? Current drug metabolism 13, 130-142.

Park, J.S., Petreas, M., Cohn, B.A., Cirillo, P.M., Factor-Litvak, P., 2009. Hydroxylated PCB metabolites (OH-PCBs) in archived serum from 1950-60s California mothers: a pilot study. Environment international 35, 937-942.

Petersen, M.S., Halling, J., Damkier, P., Nielsen, F., Grandjean, P., Weihe, P., Brosen, K., 2007. Polychlorinated biphenyl (PCB) induction of CYP3A4 enzyme activity in healthy Faroese adults. Toxicol Appl Pharmacol 224, 202-206.

Peterson, M., 2012. Hyperthyroidism in Cats: What's causing this epidemic of thyroid disease and can we prevent it?

Peterson, M.E., 2013. Feline focus: Diagnostic testing for feline thyroid disease: hyperthyroidism. Compendium (Yardley, PA) 35, E3.

Phua, D.H., Lin, C.C., Wu, M.L., Deng, J.F., Yang, C.C., 2009. Neonicotinoid insecticides: an emerging cause of acute pesticide poisoning. Clinical toxicology (Philadelphia, Pa.) 47, 336-341.

Pondugula, S.R., Dong, H., Chen, T., 2009. Phosphorylation and protein–protein interactions in PXR-mediated CYP3A repression. Expert opinion on drug metabolism & toxicology 5, 861-873.

Pugliese, M., Voslarova, E., Biondi, V., Passantino, A., 2019. Clinical Practice Guidelines: An Opinion of the Legal Implication to Veterinary Medicine. Animals (Basel) 9, 577.

Pukay, B.P., 2000. The impact of the human/companion animal/veterinarian bond on the future of the veterinary profession. Can Vet J 41, 28-29.

Purkey, H.E., Palaninathan, S.K., Kent, K.C., Smith, C., Safe, S.H., Sacchettini, J.C., Kelly, J.W., 2004. Hydroxylated polychlorinated biphenyls selectively bind transthyretin in blood and inhibit amyloidogenesis: rationalizing rodent PCB toxicity. Chemistry & biology 11, 1719-1728.

Rauert, C., Harner, T., Schuster, J.K., Eng, A., Fillmann, G., Castillo, L.E., Fentanes, O., Ibarra, M.V., Miglioranza, K.S.B., Rivadeneira, I.M., Pozo, K., Aristizábal Zuluaga, B.H., 2018. Air monitoring of new and legacy POPs in the Group of Latin America and Caribbean (GRULAC) region. Environmental Pollution 243, 1252-1262.

Rendic, S., 2002. Summary of information on human CYP enzymes: human P450 metabolism data. Drug metabolism reviews 34, 83-448.

Richardson, K.L., Schlenk, D., 2011. Biotransformation of 2,2',5,5'-tetrachlorobiphenyl (PCB 52) and 3,3',4,4'-tetrachlorobiphenyl (PCB 77) by liver microsomes from four species of sea turtles. Chemical research in toxicology 24, 718-725.

Rodriguez-Antona, C., Donato, M.T., Pareja, E., Gomez-Lechon, M.J., Castell, J.V., 2001. Cytochrome P-450 mRNA expression in human liver and its relationship with enzyme activity. Archives of biochemistry and biophysics 393, 308-315.

Ruder, A.M., Hein, M.J., Hopf, N.B., Waters, M.A., 2017. Cancer incidence among capacitor manufacturing workers exposed to polychlorinated biphenyls. American journal of industrial medicine 60, 198-207.

Rust, M.K., 2005. Advances in the control of Ctenocephalides felis (cat flea) on cats and dogs. Trends in parasitology 21, 232-236.

Sadler, N.C., Nandhikonda, P., Webb-Robertson, B.-J., Ansong, C., Anderson, L.N., Smith, J.N., Corley, R.A., Wright, A.T., 2016. Hepatic Cytochrome P450 Activity, Abundance, and Expression Throughout Human Development. Drug metabolism and disposition: the biological fate of chemicals 44, 984-991.

Schecter, A., Haffner, D., Colacino, J., Patel, K., Päpke, O., Opel, M., Birnbaum, L., 2010. Polybrominated diphenyl ethers (PBDEs) and hexabromocyclodecane (HBCD) in composite U.S. food samples. Environ Health Perspect 118, 357-362.

Schulz-Jander, D.A., Casida, J.E., 2002. Imidacloprid insecticide metabolism: human cytochrome P450 isozymes differ in selectivity for imidazolidine oxidation versus nitroimine reduction. Toxicology Letters 132, 65-70.

Selvam, V., Srinivasan, S., 2019. Neonicotinoid Poisoning and Management. Indian J Crit Care Med 23, S260-S262.

Serpe, F.P., Fiorito, F., Esposito, M., Ferrari, A., Fracassi, F., Miniero, R., Pietra, M., Roncada, P., Brambilla, G., 2018. Polychlorobiphenyl levels in the serum of cats from residential flats in Italy: Role of the indoor environment. Journal of environmental science and health. Part A, Toxic/hazardous substances & environmental engineering 53, 777-785.

Shah, S.S., Sanda, S., Regmi, N.L., Sasaki, K., Shimoda, M., 2007. Characterization of cytochrome P450-mediated drug metabolism in cats. Journal of veterinary pharmacology and therapeutics 30, 422-428.

Sheets, L.P., Li, A.A., Minnema, D.J., Collier, R.H., Creek, M.R., Peffer, R.C., 2016. A critical review of neonicotinoid insecticides for developmental neurotoxicity. Critical reviews in toxicology 46, 153-190.

Shi, X., Dick, R.A., Ford, K.A., Casida, J.E., 2009. Enzymes and inhibitors in neonicotinoid insecticide metabolism. J Agric Food Chem 57, 4861-4866.

Siddiqi, M.A., Laessig, R.H., Reed, K.D., 2003. Polybrominated diphenyl ethers (PBDEs): new pollutants-old diseases. Clin Med Res 1, 281-290.

Siller, M., Goyal, S., Yoshimoto, F.K., Xiao, Y., Wei, S., Guengerich, F.P., 2014. Oxidation of endogenous N-arachidonoylserotonin by human cytochrome P450 2U1. The Journal of biological chemistry 289, 10476-10487.

Simon-Delso, N., Amaral-Rogers, V., Belzunces, L.P., Bonmatin, J.M., Chagnon, M., Downs, C., Furlan, L., Gibbons, D.W., Giorio, C., Girolami, V., Goulson, D.,

Kreutzweiser, D.P., Krupke, C.H., Liess, M., Long, E., McField, M., Mineau, P., Mitchell, E.A.D., Morrissey, C.A., Noome, D.A., Pisa, L., Settele, J., Stark, J.D., Tapparo, A., Van Dyck, H., Van Praagh, J., Van der Sluijs, J.P., Whitehorn, P.R., Wiemers, M., 2015. Systemic insecticides (neonicotinoids and fipronil): trends, uses, mode of action and metabolites. Environmental science and pollution research international 22, 5-34.

Stapleton, H.M., Kelly, S.M., Pei, R., Letcher, R.J., Gunsch, C., 2009. Metabolism of polybrominated diphenyl ethers (PBDEs) by human hepatocytes in vitro. Environ Health Perspect 117, 197-202.

Sugiyama, S., Uno, Y., Amano, T., Kitazawa, T., Teraoka, H., 2019. Genetic diversity of cytochrome P450 2A with different metabolic activities in domestic cats. Journal of Veterinary Medical Science advpub.

Sun, Y., Wang, Y., Liang, B., Chen, T., Zheng, D., Zhao, X., Jing, L., Zhou, X., Sun, Z., Shi, Z., 2020. Hepatotoxicity of decabromodiphenyl ethane (DBDPE) and decabromodiphenyl ether (BDE-209) in 28-day exposed Sprague-Dawley rats. Science of The Total Environment 705, 135783.

Taira, K., Aoyama, Y., Kawakami, T., Kamata, M., Aoi, T., 2011. [Detection of chloropyridinyl neonicotinoid insecticide metabolite 6-chloronicotinic acid in the urine: six cases with subacute nicotinic symptoms]. Chudoku kenkyu : Chudoku Kenkyukai jun kikanshi = The Japanese journal of toxicology 24, 222-230.

Takaguchi, K., Nishikawa, H., Mizukawa, H., Tanoue, R., Yokoyama, N., Ichii, O., Takiguchi, M., Nakayama, S.M.M., Ikenaka, Y., Kunisue, T., Ishizuka, M., Tanabe,

S., Iwata, H., Nomiyama, K., 2019. Effects of PCB exposure on serum thyroid hormone levels in dogs and cats. Science of The Total Environment 688, 1172-1183.

Takahashi, K., Tsurumi, T., Inami, M., Li, Z., Kusakabe, T., Kikkawa, S., Azumaya, I., Tominaga, N., Ikenaka, Y., Arizono, K., Kato, K., 2019. Syntheses of 4-OH and 5-OH Imidacloprids. ChemistrySelect 4, 7343-7345.

Takigami, H., Suzuki, G., Hirai, Y., Sakai, S., 2009. Brominated flame retardants and other polyhalogenated compounds in indoor air and dust from two houses in Japan. Chemosphere 76, 270-277.

Tanaka, N., Miyasho, T., Shinkyo, R., Sakaki, T., Yokota, H., 2006. cDNA cloning and characterization of feline CYP1A1 and CYP1A2. Life Sciences 79, 2463-2473.

Tanaka, N., Shinkyo, R., Sakaki, T., Kasamastu, M., Imaoka, S., Funae, Y., Yokota, H., 2005. Cytochrome P450 2E polymorphism in feline liver. Biochimica et Biophysica Acta (BBA) - General Subjects 1726, 194-205.

Tehrani, R., Van Aken, B., 2014. Hydroxylated polychlorinated biphenyls in the environment: sources, fate, and toxicities. Environmental science and pollution research international 21, 6334-6345.

Temesvári, M., Kóbori, L., Paulik, J., Sárváry, E., Belic, A., Monostory, K., 2012. Estimation of Drug-Metabolizing Capacity by Cytochrome P450 Genotyping and Expression. Journal of Pharmacology and Experimental Therapeutics 341, 294-305.

Thelen, K., Dressman, J.B., 2009. Cytochrome P450-mediated metabolism in the human gut wall. The Journal of pharmacy and pharmacology 61, 541-558.

Thompson, D.A., Lehmler, H.-J., Kolpin, D.W., Hladik, M.L., Vargo, J.D., Schilling, K.E., LeFevre, G.H., Peeples, T.L., Poch, M.C., LaDuca, L.E., Cwiertny, D.M., Field, R.W., 2020. A critical review on the potential impacts of neonicotinoid insecticide use: current knowledge of environmental fate, toxicity, and implications for human health. Environmental Science: Processes & Impacts.

Thum, T., Borlak, J., 2000. Gene expression in distinct regions of the heart. The Lancet 355, 979-983.

Tomaszewski, P., Kubiak-Tomaszewska, G., Lukaszkiewicz, J., Pachecka, J., 2008. Cytochrome P450 polymorphism--molecular, metabolic, and pharmacogenetic aspects. III. Influence of CYP genetic polymorphism on population differentiation of drug metabolism phenotype. Acta poloniae pharmaceutica 65, 319-329.

Tomizawa, M., Casida, J.E., 2000. Imidacloprid, thiacloprid, and their imine derivatives up-regulate the alpha 4 beta 2 nicotinic acetylcholine receptor in M10 cells. Toxicol Appl Pharmacol 169, 114-120.

Turyk, M.E., Persky, V.W., Imm, P., Knobeloch, L., Chatterton, R., Anderson, H.A., 2008. Hormone disruption by PBDEs in adult male sport fish consumers. Environ Health Perspect 116, 1635-1641.

Uroz, F.J., Arrebola, F.J., Egea-González, F.J., Martínez-Vidal, J.L., 2001. Monitoring of 6-chloronicotinic acid in human urine by gas chromatography-tandem mass spectrometry as indicator of exposure to the pesticide imidacloprid. The Analyst 126, 1355-1358.

Uwimana, E., Maiers, A., Li, X., Lehmler, H.J., 2017. Microsomal Metabolism of Prochiral Polychlorinated Biphenyls Results in the Enantioselective Formation of Chiral Metabolites. Environ Sci Technol 51, 1820-1829.

van Beusekom, C.D., Schipper, L., Fink-Gremmels, J., 2010. Cytochrome P450-mediated hepatic metabolism of new fluorescent substrates in cats and dogs. Journal of veterinary pharmacology and therapeutics 33, 519-527.

van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N., Peterson, R.E., 2006. The 2005 World Health Organization Re-evaluation of Human and Mammalian Toxic Equivalency Factors for Dioxins and Dioxin-like Compounds. Toxicological sciences : an official journal of the Society of Toxicology 93, 223-241.

Venier, M., Hites, R.A., 2011. Flame Retardants in the Serum of Pet Dogs and in Their Food. Environmental Science & Technology 45, 4602-4608.

Verreault, J., Letcher, R.J., Sonne, C., Dietz, R., 2009. In vitro metabolism of polychlorinated biphenyls and cytochrome P450 monooxygenase activities in dietary-exposed Greenland sledge dogs. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology 150, 91-100.

Vo, D.T., Hsu, W.H., Abu-Basha, E.A., Martin, R.J., 2010. Insect nicotinic acetylcholine receptor agonists as flea adulticides in small animals. Journal of veterinary pharmacology and therapeutics 33, 315-322.

Walter, K.M., Lin, Y.P., Kass, P.H., Puschner, B., 2017. Association of Polybrominated Diphenyl Ethers (PBDEs) and Polychlorinated Biphenyls (PCBs) with Hyperthyroidism in Domestic Felines, Sentinels for Thyroid Hormone Disruption. BMC veterinary research 13, 120.

Wang, F., Wang, J., Dai, J., Hu, G., Wang, J., Luo, X., Mai, B., 2010. Comparative tissue distribution, biotransformation and associated biological effects by decabromodiphenyl ethane and decabrominated diphenyl ether in male rats after a 90-day oral exposure study. Environ Sci Technol 44, 5655-5660.

Wang, F., Wang, J., Hu, G., Luo, X., Mai, B., Dai, J., 2012. Tissue distribution and associated toxicological effects of decabrominated diphenyl ether in subchronically exposed male rats. ISRN Toxicol 2011, 989251-989251.

Wang, W., Huang, M.J., Zheng, J.S., Cheung, K.C., Wong, M.H., 2013. Exposure assessment and distribution of polychlorinated biphenyls (PCBs) contained in indoor and outdoor dusts and the impacts of particle size and bioaccessibility. The Science of the total environment 463-464, 1201-1209.

Wang, X., Banks, A.P.W., He, C., Drage, D.S., Gallen, C.L., Li, Y., Li, Q., Thai, P.K., Mueller, J.F., 2019. Polycyclic aromatic hydrocarbons, polychlorinated biphenyls and legacy and current pesticides in indoor environment in Australia - occurrence, sources and exposure risks. The Science of the total environment 693, 133588.

Watanabe, H., Sugimoto, R., Ikegami, K., Enoki, Y., Imafuku, T., Fujimura, R., Bi, J., Nishida, K., Sakaguchi, Y., Murata, M., Maeda, H., Hirata, K., Jingami, S., Ishima, Y., Tanaka, M., Matsushita, K., Komaba, H., Fukagawa, M., Otagiri, M., Maruyama,

T., 2017. Parathyroid hormone contributes to the down-regulation of cytochrome P450 3A through the cAMP/PI3K/PKC/PKA/NF-κB signaling pathway in secondary hyperparathyroidism. Biochemical Pharmacology 145, 192-201.

Whitehead, T.P., Brown, F.R., Metayer, C., Park, J.-S., Does, M., Dhaliwal, J., Petreas, M.X., Buffler, P.A., Rappaport, S.M., 2014. Polychlorinated biphenyls in residential dust: sources of variability. Environmental science & technology 48, 157-164.

Wood, T.J., Goulson, D., 2017. The environmental risks of neonicotinoid pesticides: a review of the evidence post 2013. Environmental science and pollution research international 24, 17285-17325.

Wu, X., Kammerer, A., Lehmler, H.J., 2014. Microsomal oxidation of 2,2',3,3',6,6'-hexachlorobiphenyl (PCB 136) results in species-dependent chiral signatures of the hydroxylated metabolites. Environ Sci Technol 48, 2436-2444.

Xu, M., Ju, W., Hao, H., Wang, G., Li, P., 2013. Cytochrome P450 2J2: distribution, function, regulation, genetic polymorphisms and clinical significance. Drug metabolism reviews 45, 311-352.

Xue, L., Zgoda, V.G., Arison, B., Almira Correia, M., 2003. Structure–function relationships of rat liver CYP3A9 to its human liver orthologs: site-directed active site mutagenesis to a progesterone dihydroxylase. Archives of biochemistry and biophysics 409, 113-126.

Yoo, J., Hirano, M., Mizukawa, H., Nomiyama, K., Agusa, T., Kim, E.-Y., Tanabe, S., Iwata, H., 2015. In Vitro and in Silico Analyses for Predicting Hepatic Cytochrome P450-

Dependent Metabolic Potencies of Polychlorinated Biphenyls in the Baikal Seal. Environmental Science & Technology 49, 14588-14596.

Yoon, E., Babar, A., Choudhary, M., Kutner, M., Pyrsopoulos, N., 2016. Acetaminophen-Induced Hepatotoxicity: a Comprehensive Update. Journal of clinical and translational hepatology 4, 131-142.

Zanger, U.M., Schwab, M., 2013. Cytochrome P450 enzymes in drug metabolism: Regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacology & Therapeutics 138, 103-141.

Zhang, Q.-Y., Dunbar, D., Ostrowska, A., Zeisloft, S., Yang, J., Kaminsky, L.S., 1999. Characterization of Human Small Intestinal Cytochromes P-450. Drug Metabolism and Disposition 27, 804.

Zhang, X., Su, T., Zhang, Q.-Y., Gu, J., Caggana, M., Li, H., Ding, X., 2002. Genetic Polymorphisms of the Human CYP2A13 Gene: Identification of Single-Nucleotide Polymorphisms and Functional Characterization of an Arg257Cys Variant. Journal of Pharmacology and Experimental Therapeutics 302, 416.

Zuber, R., Anzenbacherova, E., Anzenbacher, P., 2002. Cytochromes P450 and experimental models of drug metabolism. Journal of cellular and molecular medicine 6, 189-198.

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Abstract

Pet cats are frequently exposed to veterinary drugs and a variety of environmental compounds. They are also known to be especially sensitive to some drugs and chemical exposures. To identify their species' sensitivity and toxicity caused by these environmental exposures, the knowledge on biotransformation ability for several xenobiotics in cats is thus required. Cytochrome P450 (CYP) is one of the most dominant metabolism enzymes in phase I and can be induced by numerous compounds. The study on feline CYP isozymes expression involved in chemical exposures is necessary for the prediction of adverse effects forward to drug development and veterinary clinic medication. In this study, polychlorinated biphenyls (PCBs), polybrominated diphenyl ethers (PBDEs) and neonicotinoids are selected as the models of environmental compounds exposed to domestic cats worldwide. This present study aimed to elucidate the mRNA expression of the CYP1–CYP3 families in the cat tissues and CYP mRNA expression related to PCB and PBDE exposures, and to investigate the species differences in CYP involved in metabolism of PCBs and neonicotinoids between cats and other species. The *in vivo* exposures and *in vitro* CYP metabolism assay were conducted. Our results found that, in cats, the greatest abundance of CYP1–CYP3 (CYP1A2, CYP2A13, CYP2C41, CYP2D6, CYP2E1, CYP2E2, CYP2F2, CYP2F5, CYP2J2, CYP2U1, and CYP3A132) was expressed in the liver, but some extrahepatic isozymes were found in the kidney (CYP1A1), heart (CYP1B1), lung (CYP2B11 and CYP2S1) and small intestine (CYP3A131). Feline CYP1A1, CYP1A2 and CYP1B1 were significantly upregulated in the liver as well as in several tissues after once exposure to PCBs. However, these CYP1–CYP3 showed no significant difference in mRNA expression between control and BDE-209

exposure cats indicate that the chronic exposure of BDE-209 could not change CYP expression in the liver of cats. The study of *in vitro* CYP-mediated PCB metabolism found that the OH-PCB profiles between cats and dogs were similar and 4′OH-CB18 was major metabolite. These findings combined with *in silico* docking simulation indicated that cat CYP3A and dog CYP3A/1A1 could mainly catalyze most PCBs, particularly PCB18, while CYP1A1 in cats and CYP1A2/2B in dogs may be less players for the metabolism of some PCBs. The levels of OH-PCB formation indicate feline CYPs have lower affinity to PCBs than those in dogs. The kinetic parameters of CYP metabolizing neonicotinoids indicate cats have particularly low CYP activity for metabolism of neonicotinoids in comparison to rats and humans. The feline glucuronidation deficiency together with all our findings suggested that PCBs and neonicotinoids may be metabolized less in cats as compared to other species.