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Evaluation of anticoagulant rodenticide sensitivity by examining *in vivo* and *in vitro* responses in avian species, focusing on raptors☆

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A B S T R A C T

Anticoagulant rodenticides (ARs) are used to control pest rodent species but can result in secondary poisoning of non-target animals, especially raptors. In the present study, differences in AR sensitivity among avian species were evaluated by comparing *in vivo* warfarin pharmacokinetics and effects, measuring cytochrome P450s (CYPs) expression involved in AR metabolism, and conducting *in vitro* inhibition assays of the AR target enzyme Vitamin K 2,3-epoxide reductase (VKOR). Oral administration of warfarin at 4 mg/kg body weight did not prolong prothrombin time in chickens (*Gallus gallus*), rock pigeons (*Columba livia*), or Eastern buzzards (*Buteo japonicus*). Rock pigeons and buzzards exhibited shorter plasma half-life of warfarin compared to chickens. For the metabolite analysis, 4′-hydroxywarfarin was predominantly detected in all birds, while 10-hydroxywarfarin was only found in pigeons and raptors, indicating interspecific differences in AR metabolism among birds likely due to differential expression of CYP enzymes involved in the metabolism of ARs and variation of VKOR activities among these avian species. The present findings, and results of our earlier investigations, demonstrate pronounced differences in AR sensitivity and pharmacokinetics among bird species, and in particular raptors. While ecological risk assessment and mitigation efforts for ARs have been extensive, AR exposure and adverse effects in predatory and scavenging wildlife continues. Toxicokinetic and toxicodynamic data will assist in such risk assessments and mitigation efforts.

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1. Introduction

Rodents are globally distributed, and inhabit agricultural, rural and urban areas. Commensal rodents, including black rats (*Rattus rattus*) and brown rats (*Rattus norvegicus*), are sources of numerous pathogens that cause serious diseases and death in humans including zoonotic bacteria such as *Leptospira interrogans*, responsible for Leptospirosis, and *Yersina pestis*, responsible for plague (Himsworth et al., 2013). They also cause economic damage by eating crops in agricultural areas, consuming and fouling food stores, and gnawing electrical cables that disrupt power supply in urban areas (van den Brink et al., 2018). Although rodents play an important role in many ecosystems, invasive rodents are sometimes harmful. In some island ecosystems, invasive rodents eat native plants and animals, ultimately changing species compositions and destroying unique ecosystems (Shiels et al., 2014). Thus, the control and even eradication of rodents in some settings has become commonplace and essential.

Rodent control can take many forms including physical, chemical, biological and exclusionary processes (Witmer, 2018). Since the coumarin derivative was developed as an anticoagulant rodenticide (AR) in 1948 (Last, 2002), warfarin and other ARs have been used in 77% of measures for control of invasive rodents over the past 75 years with varying degrees of success (Duron et al., 2017). ARs, such as warfarin and diphacinone, inhibit their pharmacological target, Vitamin K 2,3-epoxide reductase (VKOR), which reduces Vitamin K 2,3-epoxide (VKO) to Vitamin K, impeding the activation of clotting factors II, VII, IX, and X (Rishavy et al., 2018; Watt et al., 2005). This results in internal hemorrhage and eventual death of target rodents.

ARs are very useful and effective for pest control, however there are many reports about exposure and adverse effects in non-target species. Exposure and potentially toxic effects occur when non-target animals directly consume AR bait (primary exposure) or indirectly consume poisoned rodents and unintentionally expose prey (secondary exposure), where pest control has been implemented (van den Brink et al., 2018). Predatory and scavenging birds and mammals are exposed and affected on a global scale. A news article entitled “Killing Rats is Killing Birds” in Nature News reported that more than 130 raptors were found dead with rodenticide residue in their liver in Vancouver, Canada, and gained great attention worldwide (Lovett, 2012). In Scotland, black kites (*Milvus migrans*) were found to be particularly vulnerable to AR exposure, with about 70% containing residues, and 10.5% having died as a result of rodenticide ingestion (Hughes et al., 2013). In Taiwan, 61.5% of 221 raptor samples contained ARs, with many exceeding a toxicity threshold of 0.1 mg/kg liver, and 37.8% of 66 AR-exposed kites having liver residues >0.2 mg/kg (Hong et al., 2019). Thus, damage caused by ARs to non-target animals, especially raptors, has been widely described, and seemingly continues in developed countries despite efforts to mitigate risk.

Various ARs have been used in Japan, including warfarin with its application initiated in the late 1940s. 4-hydroxycoumarin derivatives, such as warfarin and coumatetralyl, and indanediones, such as diphacinone and chlorophacinone, are sometimes categorized as first-generation anticoagulant rodenticides (FGARs) (Mercer et al., 2022; Rattner and Mastrota, 2018). However, since the 1950s, there have been reports of resistance to these rodenticides by wild rodents (Berny et al., 2018; Buckle, 2013). Thus, second-generation compounds were developed in an effort to overcome resistance to FGARs. Second-generation anticoagulant rodenticides (SGARs), commercialized in the 1970s, are more potent and can result in lethality with merely a single feeding in target rodents. SGARs include brodifacoum, bromadiolone, difenacoum, and difethialone (Eason et al., 2002). However, the Agricultural Chemicals Regulation Law in Japan prohibits SGAR use.

Assessment of the sensitivity of wild birds to various ARs, and the exposure susceptibility of birds inhabiting AR-treated areas is essential for safe rodent control. In Japan, AR use has been conducted without sufficient evaluation of the effects of AR exposure on non-target animals. For example, in northern Japan on Yururi and Moyururi Islands, invasive rodent control using diphacinone was conducted between 2013 and 2016. In the Ogasawara islands, located to the south of the mainland Japan, invasive rodents had been reported, and widespread application of diphacinone pellets by helicopter and in bait stations was started and continue as activities. However, data on the sensitivity and exposure to ARs for the islands’ endemic species, such as buzzard (*Buteo japonicus toyoshimai*) and pigeon (*Columba janthina nitens*), are lacking.

In avian species, the median lethal dose (LD_{50}) for ARs and the median lethal dietary concentration (LC_{50}) of ARs have been estimated based on common test species, such as the chicken (*Gallus gallus*), bobwhite (*Colinus virginianus*), Japanese quail (*Coturnix japonica*) and mallard (*Anas platyrhynchos*). The estimated LD_{50} of warfarin is 942 mg/kg for chickens and >2150 mg active ingredient/kg for bobwhite quail, doses which are generally higher (i.e. less toxic) than those observed in the laboratory rat (*Rattus norvegicus*) (Erickson and Urban, 2004; Rattner and Mastrota, 2018). There is evidence that the toxicity of several ARs in birds is markedly lower than in mammals, but with some exception (Rattner and Mastrota, 2018). For example, raptors including American kestrels (*Falco sparverius*) and Eastern screech-owls (*Megascops asio*) seem to be more sensitive to diphacinone than common avian test species (Rattner et al., 2012; Rattner et al., 2011). Even though ARs have been detected and linked to mortality in many avian species, especially raptorial and scavenging birds, few studies have directly compared AR sensitivity among avian species.

In general, sensitivity to chemicals varies among species, individuals, and their sex, age, reproductive state, and physical condition. These differences are determined by various factors including the affinity of the chemical to its target enzyme and pharmacokinetics processes (absorption, distribution, metabolism, excretion; ADME). Sensitivity to ARs is affected by their affinity to the target enzyme of VKOR and metabolism by cytochrome P450 (CYP) superfamilies. In humans, the most impactful factors for warfarin sensitivity are single nucleotide polymorphisms (SNP) in VKORC1 (Vitamin K epoxide reductase complex subunit 1) (Yuan et al., 2005) and CYP2C9 (Pirmohamed, 2006; Wadelius et al., 2007), and determine the warfarin dose for each patient that will help prevent venous thrombosis. In rats, genetic changes in VKORC1 and CYPs led to AR resistance. For example, warfarin-resistant rats found in Tokyo, Japan have been shown to have increased expression of CYP2B1, 2C6, 2C11, and 3A2, responsible for warfarin metabolism in the liver (Takeda et al., 2016). In addition, these rats had a VKOR with a mutation of leucine at position 76 to a proline, which is within the endoplasmic reticulum-luminal loop, and had a 37.5-times higher IC_{50} value for warfarin compared to susceptible rats (Takeda et al., 2021).

A previous report (Watanabe et al., 2015) showed that the cumulative intrinsic clearance of warfarin was 4.3 mL/min/nmol CYP in rats, whereas it was 36.6 in large-billed crows (*Corvus macrorhynchos*) and 24.5 in chickens. In addition, another *in vitro* study was conducted on several avian species, including both poultry (chicken, and turkey, *Meleagris gallopavo*) and raptors, to compare the VKOR enzymatic activity. The results demonstrated that owls (snowy owl; *Bubo scandiacus* and great horned owl; *Bubo virginianus*), hawks (sparrowhawk; *Accipiter nisus nisosimilis* and goshawk; *Accipiter gentilis fujiyamae*), and peregrine falcon (*Falco peregrinus pealei*) had lower VKOR enzymatic activity that was inhibited by warfarin more readily than in chickens and turkeys (Nakayama et al., 2020). These results indicate that pronounced interspecific differences in AR sensitivity exist among avian species, but the findings are principally based on *in vitro* studies. The objective of the present study was to further evaluate differences in AR sensitivity among birds, including several species of raptors, by *in vivo* studies on warfarin pharmacokinetics and effects on clotting time, differences in CYP gene expression that may affect AR metabolism, and also by *in vitro* inhibition of VKOR. This approach will provide different and complementary information to better understand the basis of AR sensitivity in non-target avian species.

2. Materials and methods

2.1. Chemicals

Chemicals used in this study are shown in Supporting Information (SI).

2.2. Animals

Details of the study type, animal biometrics and sources, exposure route and sample collection are summarized in Table S1. The *in vivo* experiments were performed at (1) Kasetsart University Raptor Rehabilitation Unit (Thailand) in 2019, (2) Hokkaido University in 2020 and (3) the Institute for Raptor Biomedicine Japan; IRBJ (Kushiro, Japan) in 2020 and 2021.

Predatory birds used in the studies conducted at Kasetsart University Raptor Rehabilitation Unit (Thailand) included Brahminy kite (*Haliastur indus,* n = 4), Black kite (*Milvus migrans,* n = 2), Crested serpent eagle (*Spilornis cheela,* n = 2), Changeable hawk eagle (*Nisaetus limnaeetus,* n = 2), Eastern barn owl (*Tyto javanica,* n = 1), Black-winged kite (*Elaneus caeruleus,* n = 2), Himalayan buzzard (*Buteo burmanicus,* n = 1) and Collared scops owl (*Otus lettia,* n = 5). They were housed outdoors under a natural lighting cycle at ambient temperature and fed dead day-old chicks every other day.

Domestic birds used in the study performed at Hokkaido University (Japan) included chickens (*Gallus gallus*, n = 10, Farm at Hokkaido University, Sapporo, Japan) and rock pigeons (*Columba livia*, n = 10, local breeder farm, Hokkaido). They were maintained indoors at 22 °C in a 12/12 h light/dark cycle and, fed a commercial dry food (grains: 72.4%, vegetable oil cake: 19.4%, other: 8.2%) and water ad libitum.

Eastern buzzards (*Buteo japonicus,* n = 2) were used in the *in vivo* study conducted at IRBJ (Kushiro, Japan). They were housed outdoors under a natural lighting cycle at ambient temperature and were fed commercially available house mice (*Mus musculus*) once a day.

For the *in vivo* experiment conducted at the Faculty of Veterinary Medicine, Hokkaido University and IRBJ, all the animal care and experimental procedures were approved and performed in accordance with the guidelines of the Animal Care and Use Committee of Hokkaido University (approval numbers: 19-0033, 20-0053 and 20-0136). Since 2007, the Faculty of Veterinary Medicine, Hokkaido University has obtained full accreditation from of the American Association for Laboratory Animal Care (AAALAC) International (Frederick, Maryland, USA). For the *in vivo* experiment conducted in Thailand, the animal use protocol was submitted and approved by the Kasetsart University Institutional Animal Care and Use Committee (approval number: ACKU62-VET-051) and found to be in accordance with the guidelines of animal care and use under the Ethical Review Board of the Office of National Research Council of Thailand (NRCT) for the conduct of the scientific research.

2.3. Warfarin administration and blood collection

Before warfarin administration, animals were fasted for 12 h. Warfarin sodium was dissolved in distilled water and the solution was administered orally to the birds (4 mg/kg body weight, n = 5 chickens and pigeons, n = 2 buzzards, and other raptors; Table S1). This oral dose is sublethal, corresponding to 0.0042 of the median lethal dose in chickens (Erickson and Urban, 2004). Intravenous administration via the basilic vein was also conducted on chickens and pigeons (n = 5, respectively) to compare the results with that of oral administration. Blood samples (~500 μL) were taken from the basilic vein or the medial metatarsal vein using 1 mL syringes with 25 G needles (Terumo) containing 50 μL of 3.2% sodium citrate before and after administration (collection times are shown in Table S1). Plasma was separated from each sample by centrifugation at 1500 g for 10 min and stored at −80 °C. After blood collection at 72 h post-warfarin administration, chickens and pigeons were euthanized with carbon dioxide. The liver from each bird was immediately removed, frozen in liquid nitrogen, and kept in −80 °C until use for enzymatic activity assays.

2.4. Warfarin extraction from plasma and liver

Warfarin and hydroxylated warfarin were extracted from plasma and liver by liquid-liquid extraction as previously reported (Takeda et al., 2016). Approximately 50 mg of liver or 10 μL of plasma was added to centrifuge tubes including 2 mL of 0.1 M sodium acetate (pH 5), 100 μL of 0.1 μM glucuronidated oxazepam as an indicator of deconjugation, 100 μL of 0.2 μM phenyl-d5-7-hydroxywarfarin as an internal standard and 100 μL of β-glucuronidase (>4500 unit). The mixtures in the tubes were incubated at 37 °C for more than 5 h. After incubation, 5 mL of diethyl ether was added to the tubes and vortexed for 10 min and centrifuged at 3000 g for 10 min, and the organic layer including warfarin and hydroxylated warfarin was transferred to spitz tubes and this procedure was repeated. The organic solution was evaporated to dryness by gassing with nitrogen. The residue was dissolved with 200 μL of MeOH and dispensed into an HPLC vial. The concentrations of warfarin and hydroxylated warfarin were quantified using high-performance liquid chromatography coupled with mass spectrometry (HPLC/MS) as described below.

2.5. Prothrombin time assay

Prothrombin time (PT) reflects the time of extrinsic and final common pathways of blood coagulation (Winter et al., 2017). An excess of thromboplastin promotes the cascade to begin with the activation of Factor VII which is a Vitamin K-dependent clotting factor. PT was measured in citrated plasma samples of chickens and buzzards after *p.o.* and *i.v.* administration of 4 mg/kg warfarin as previously reported (Rattner et al., 2010). However, PT values for pigeons and other raptors were excluded due to insufficient sample volume and/or inadequate fibrinogen concentration.

Crude chick hatchling thromboplastin (CHT) was prepared as described in Rattner et al. (2010) (the details in SI). The CHT was suspended in 25 mM $CaCl_2$ (volume ratio of 50 mg CHT/2500 μL 25 mM $CaCl_2$), incubated at 42 °C for 15 min, and gently vortexed every 3 min during incubation. The CHT suspension was centrifuged at 1500 g for 20 min. The supernatant was transferred to tubes in ice and diluted with 25 mM $CaCl_2$ at the same volume. Before the PT measurement, the CHT solution was incubated at 37 °C for 2–3 min, 50 μL of plasma was added into cuvettes and incubated at 37.4 °C for 1 min. The measurement of PT was started automatically with the addition of 100 μL of the CHT solution using the Sysmex® CA-104 (Sysmex Corporation, Kobe, Japan). PT was determined by detecting fibrinogen clots as the change of transmitted light. Instrument precision of the PT assay was evaluated using human plasma reference Dade® Ci-Trol® Level 1 as control plasma and Dade® Ci-Trol® Level 2 as abnormal plasma every time before analysis with Thromborel® S (Siemens Healthcare Diagnostics Products GmbH), a human placenta thromboplastin, as an initiator of reaction. Although the volume of some samples was limited, most samples were assayed in duplicate.

In addition to PT assay, the thrombin clotting time assay (TCT) was conducted to ensure that adequate fibrinogen was present to promote clot formation in each sample. The assay method is described in SI. PT values of some samples were excluded because fibrinogen concentration was very low or not detected, likely due to improper sample collection or processing.

2.6. CYP gene sequences and their inferred expression levels of buzzard by RNA-sequencing analysis (RNA-seq)

Total RNA was extracted from the liver of the Ogasawara's buzzard as previously described (Nakayama et al., 2020). The RNA-seq was

performed by Hokkaido System Science Co., Ltd. (Sapporo, Hokkaido, Japan). For RNA-seq, RNA concentration measurements and quality checks were performed using Nanodrop (Thermo Fisher Scientific) and Agilent 2100 Bioanalyzer (Agilent Technologies, CA, USA) respectively. The library preparation for sequencing was performed using TruSeq Strand mRNA Sample Prep Kit (Illumina, CA, USA) according to the manufacturer's protocol. The libraries were sequenced using a HiSeq 2500 (Illumina) as paired-end 101-bp reads.

For removing adapter sequences, cutadapt ver. 1.18 (Martin, 2011) was used followed by removing poly-A sequences, low quality (<20), and short (<30 bp) sequences using prinseq (Schmieder and Edwards, 2011). Trinity ver. 2.8.4 (Grabherr et al., 2011) was used to assemble the trimmed short reads. To annotate the assembly results, BLASTN was performed using the assembly results as a query and the RefSeq RNA database of the chicken genome (assembly accession: GCF_000002315.5) as a database.

The sequences annotated as CYP1-3 family from the assembly results were used as a reference for re-mapping analysis using Hisat2 ver.2.1.0 (Kim et al., 2015). The Samtools ver. 1.5 (Li et al., 2009) was used to sort and to then cufflinks ver. 2.2.1 (Trapnell et al., 2010) was used to count the mapped reads.

2.7. Liver collection and preparation of microsomes

The information on birds used for *in vitro* studies is shown in Table S1. Liver samples were collected from chickens (n = 10), pigeons (n = 10) and wild birds (moribund animals or those that recently died) including Blakiston's fish owls (*Ketupa blakistoni,* n = 3, IRBJ), buzzard (n = 1, Maruyama Zoo), and an Ogasawara's buzzard (*Buteo japonicus toyoshimai*, n = 1, Institute of Boninology). These liver samples were frozen in liquid nitrogen and stored at −80 °C until use for the preparation of liver microsomes as previously described (Nakayama et al., 2020; SI).

2.8. In vitro VKOR activity inhibition test

The VKOR activity and inhibition tests were performed as previously reported (Yamamura et al., 2021). The liver microsome fraction was diluted with 0.1 M HEPES buffer (pH 7.4) and adjusted to a final concentration of 1 mg/mL with a total volume of 100 μL. The mixtures contained Vitamin K1 epoxide (0.1 mM) as substrate. For the VKOR inhibition studies, warfarin concentrations of 0, 0.1, 0.5, 1, 5, 10, 25 μM and diphacinone concentrations of 0, 0.1, 0.25, 0.5, 1.0, 2.5, 10 μM were included in each study. Before reactions, the mixtures were pre-incubated for 5 min. Reactions were initiated by the addition of THP (final concentration 1 mM) and continued for 20 min and stopped by the addition of 1 mL of diethyl ether. The preincubation and reaction temperatures were set depending on body temperatures of each bird at 42 °C for chickens, 41 °C for pigeons, and 40.5 °C for Blakiston's fish owl (Rashotte et al., 1995). After terminating each reaction, 10 μL of 0.1 μM Vitamin K1-d7 as an internal standard and 1 mL of 0.1 M HEPES buffer were added to the mixture. Vitamin K and VKO were extracted from the mixture by liquid-liquid extraction method. 5 mL of diethyl ether was added to the mixtures, vortexed for 10 min, and centrifuged at 3000 g for 10 min. The organic layer including Vitamin K and VKO was transferred to spitz tubes and this procedure was repeated. The organic solution was evaporated to dryness by gassing with nitrogen. The residue was dissolved with 100 μL of MeOH and dispensed into HPLC vial. The concentration of Vitamin K was quantified using HPLC/MS.

2.9. HPLC/MS conditions for quantification of warfarin and vitamin K

2.9.1. Warfarin and hydroxy warfarin

Warfarin and 4'-, 6-, 7-, 8-, 10-hydroxy warfarin were separated using a reversed-phase C18 column (Symmetry Shield, RP18 2.1 × 150 mm, 3.5 μm) (Waters, Milford, MA). The column temperature was set at 50 °C. Samples were analyzed with high-performance liquid chromatography (HPLC) coupled with electrospray ionization ion-trap tandem mass spectrometry (ESI/MS/MS, LTQ Orbitrap, LC-8030; Shimadzu, Kyoto, Japan). The injection volume of samples was 10 μL. Mobile phase was 10 mM ammonium acetate in 10% MeOH (pH 5.0) as A, and 100% MeOH as B. The flow of the mobile phase was 0.25 mL/min, for 20 min. The time course of the gradient of mobile phase B was 20% of mobile phase B from at 0–2 min, linear gradient from 20% (2 min) to 90% (15 min), 90% of mobile phase B from 15 to 17 min, and return to 20% from 17 to 20 min. The collision energies (CE) and other MS parameters are shown in Table S2.

2.9.2. Vitamin K

Vitamin K was separated using a reversed-phase C18 column (Inertsil ODS-3, 2.1 × 150 mm, 5.0 μm) (GL Science, Tokyo, Japan). The column temperature was set at 45 °C. Samples were analyzed using HPLC coupled with atmospheric pressure chemical ionization triple quadrupole mass spectrometry (APCI/MS/MS, LC-8040; Shimadzu). The injection volume of samples was 10 μL. The mobile phase was 0.1% acetic acid in 95% MeOH as A and 100% EtOH as B. The flow of the mobile phase was 0.25 mL/min, time of measurement per sample was 10 min. The gradient of mobile phase was as shown below: linear gradient from 0% (2 min) to 100% (7 min) and return to 0% from 9.5 to 10 min. The collision energies (CE) and other MS parameters are shown in Table S3.

2.10. Quality control & quality assurance

Details of the Vitamin K and hydroxy warfarin quantification methods, recovery values, the limit of detection (LOD) and the limit of quantification (LOQ) are presented in SI.

2.11. Data analysis

2.11.1. Pharmacokinetic analysis

Non-compartment analysis for plasma concentration of warfarin and its metabolites was performed using PKPlus 2.5 (Simulations Plus, Inc. Lancaster, CA, USA). The pharmacokinetic parameters estimated include the peak plasma concentration; C_{max} (ng/mL), concentration-time area under the curve; AUC (μg*h/mL), clearance; CL (mL/h) and plasma half-life; $T_{1/2}$ (h).

2.11.2. Statistical analysis

Statistical analysis was conducted with JMP software (version 16; SAS Institute, Cary, NC, USA). All data were tested for normality using the Shapiro-Wilk test and checked for homogeneity of variance using the Brown-Forsythe test. The Dunnett's test was performed for PT of each time point. The Student's t-test was performed to compare the warfarin concentration in liver between chickens and pigeons. In order to compare differences in the pharmacokinetic parameters (AUC, Cmax, $T_{1/2}$, and CL) among the chickens, pigeons, and raptors, the raptorial species were combined into the following four groups: (1) Buzzards; Buzzard and Himalayan Buzzard, (2) Eagles; Crested Serpent Eagle and Changeable Hawk Eagle, (3) Kites; Brahminy Kite, Black Kite and Black-winged Kite, (4) Owls; Eastern Barn Owl and Collared Scops Owl. After the pharmacokinetic parameters were log-transformed, Tukey's test was performed to compare the data among the groups. In addition, a Principal Component Analysis (PCA), a multivariate technique that analyzes data to display the pattern of similarity of the observations and of the variables as points in plots, was performed. Data are presented as mean ± standard deviation (SD), and the significance level was set at $\alpha = 0.05$.

VKOR activity values were fitted by nonlinear regression with inhibition curve. Estimations of apparent IC_{50} values were obtained using the GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA). The results of this part are shown by comparison with the black rat (*Rattus rattus*) from the previous studies (Takeda et al., 2022; Watanabe et al., 2010).

3. Results

3.1. Pharmacokinetics of warfarin

Fig. 1 shows the plasma concentration-time profile of warfarin in chickens, pigeons, and buzzards after oral administration of 4 mg/kg warfarin. In chickens, the concentration of warfarin peaked at 29.6 ± 3.3 μg/mL 4 h post-administration and decreased gradually to 15.3 ± 1.3 μg/mL by 72 h (end of experiment). In pigeons, plasma warfarin reached a maximum concentration of 11.5 ± 4.3 μg/mL at 1 h post administration, but in contrast to chickens, was nearly undetectable (0.6 ± 0.3 μg/mL) after 24 h. Similar to pigeons, plasma warfarin in buzzards reached a maximum concentration of 16.1 ± 1.4 μg/mL at 2 h, and it was almost undetectable by 24 h (1.5 ± 1.4 μg/mL). For both chickens and pigeons, there was no difference in plasma warfarin concentration between oral (*per os*; *p.o.*) and intravenous (*i.v.*) exposure routes.

Among analyzed warfarin metabolites, 4′- and 10-hydroxy warfarin were found in these plasma samples, whereas 6-, 7- and 8-hydroxy warfarin could not be detected. The time course of changes in plasma 4′- and 10-hydroxy warfarin concentration is presented in Figs. 2 and 3A and B. 4′-hydroxy warfarin was the major metabolite found in chickens, pigeons and buzzards. In chickens, the concentration of 4′-hydroxy warfarin peaked at 4 h (1.3 ± 0.1 μg/mL) and levels were sustained through 72 h, while concentrations peaked at 1 h (2.1 ± 0.9 μg/mL) in pigeons and at 6 h (1.1 ± 0.5 μg/mL) in buzzards and decreased by 72 h. 10-hydroxy warfarin was detected in the plasma of pigeons (0.1–0.4 μg/mL) and buzzards (0.1–0.2 μg/mL) and levels were then sustained through 72 h, but it was barely detectable in chickens (<0.1 μg/mL) by 12 h.

Figs. S3 and S4 present the concentrations of warfarin and its metabolite residues in the liver of chickens and pigeons. Warfarin concentration was significantly higher in chickens (51.1 ± 17.2 μg/g for *p.o.* administration and 83.5 ± 13.9 μg/g for *i.v.* administration) than in pigeons (4.3 ± 2.4 μg/g for *p.o.* administration and 5.5 ± 6.0 μg/g for *i.v.* administration) (Wilcoxon test, $p = 0.01$). The composition of metabolites in the liver was dominated with 4′-hydroxy warfarin in chickens, while 10-hydroxy warfarin was the principal metabolite in pigeons.

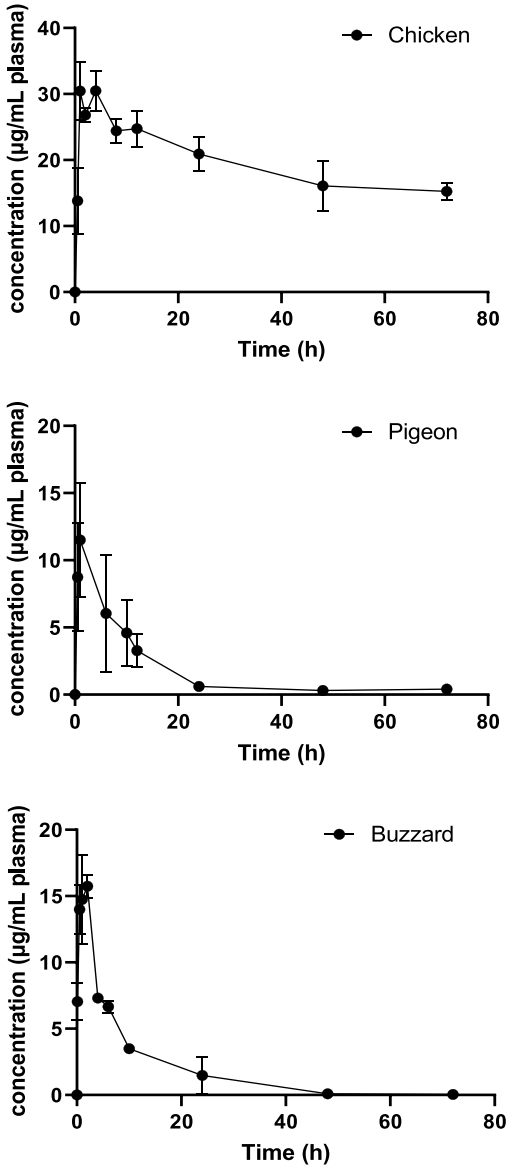

Fig. 1. Time course of changes in plasma warfarin concentration Plasma warfarin concentration in chickens (n = 5), pigeon (n = 5) and buzzard (n = 2) after oral warfarin administration at a dose of 4 mg/kg. Data are shown as mean ± SD.

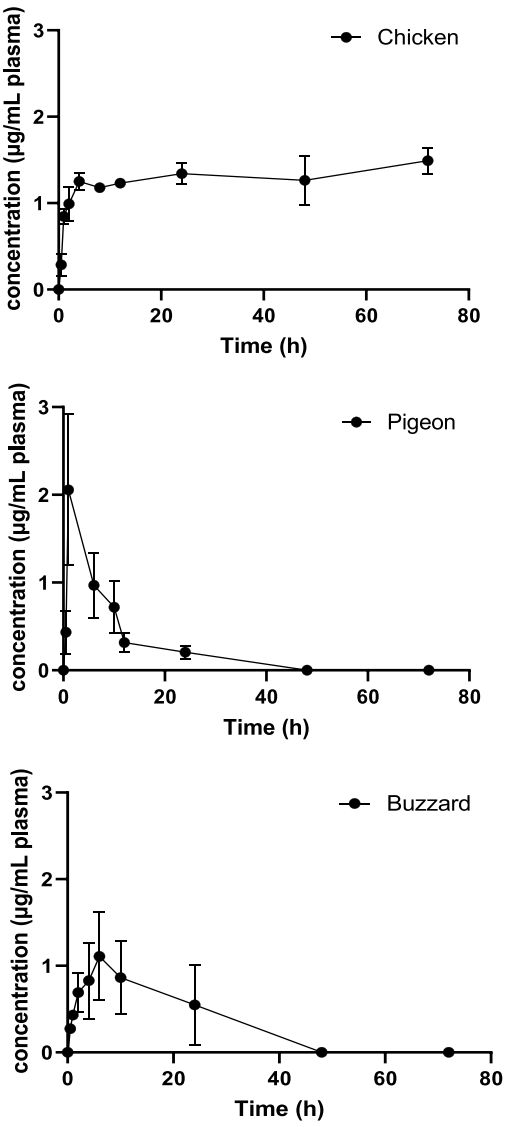

Fig. 2. Time course of changes in plasma 4′-hydroxy warfarin concentration Plasma 4′-hydroxy warfarin concentration in chickens (n = 5), pigeons (n = 5) and buzzards (n = 2) after oral warfarin administration at a dose of 4 mg/kg. Data are shown as mean ± SD.

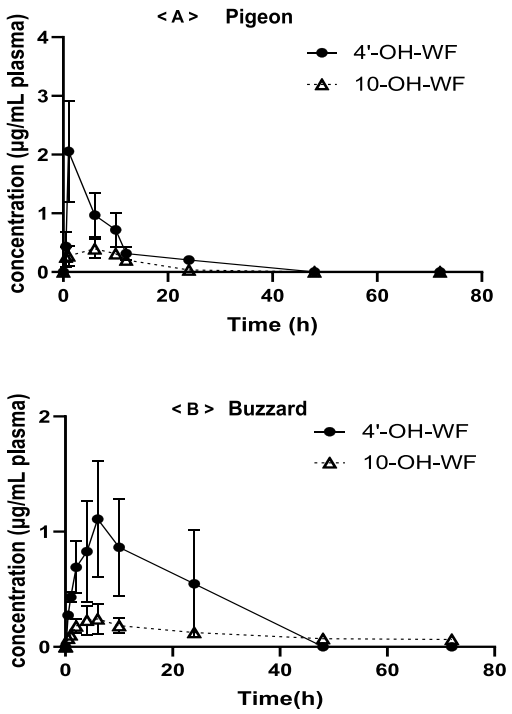

Fig. 3. A and B Time course of changes in plasma hydroxy warfarin concentration Plasma 4′- and 10-hydroxy warfarin concentration in pigeon and buzzard after oral warfarin administration at a dose of 4 mg/kg. Data are shown as mean ± SD.

Fig. S5 shows the changes in plasma warfarin concentration over time in other raptors after oral administration of 4 mg/kg warfarin. The maximum concentration was 78.3 ± 49.2 μg/mL in black kite, with C_{max} ranging from 11.5 μg/mL to 62.2 μg/mL in the 7 other species. Warfarin concentration decreased rapidly and was generally undetectable after 72 h in these species. For metabolites of warfarin, 4′-hydroxy warfarin was detected in all species (Fig. S6A), but 10-hydroxy warfarin was only detected in kites and eagles (Fig. S6B).

The pharmacokinetic parameters AUC, C_{max}, $T_{1/2}$, and CL are presented in Table 1. In pigeons (*p.o.* administration), blood collection times (0, 0.5, 1, 6, 10, 12, 24, 48, and 72 h) did not include the sampling time when the blood concentration was predicted to be greatest (3.9 h, data not shown) by PKPlus 2.5 software, and thus could have a shorter half-life than estimated. AUC in chickens (1398.4 ± 169.7 μg*h/mL), eagles (1342.3 ± 276.1 μg*h/mL) and kites (833.7 ± 355.5 μg*h/mL) was significantly higher than those in owls (415.9 ± 289.8 μg*h/mL), buzzards (254.1 ± 198.9 μg*h/mL) and pigeons (138.7 ± 37.9 μg*h/mL). C_{max} was significantly greater in owls (57.5 ± 27.5 μg/mL), kites (55.3 ± 29.6 μg/mL), eagles (39.7 ± 9.0 μg/mL) and chickens (29.6 ± 3.3 μg/mL) than in pigeons (11.5 ± 4.3 μg/mL). The most distinct difference among groups of birds was $T_{1/2}$, with the value in domestic chickens (97.9 ± 24.0 h) being many folds greater than other groups (11.8–27.8 h). CL was significantly larger in buzzards (17.8 ± 10.0 mL/h) and pigeons (12.7 ± 2.9 mL/h) than in kites (2.5 ± 1.3 mL/h) and owls (2.3 ± 3.2 mL/h).

Table 1
Pharmacokinetic parameters for warfarin in plasma of avian species following a single oral dose of 4 mg/kg.

Group	N	AUC (μg*h/mL)	C_{max} (μg/mL)	$T_{1/2}$ (h)	CL (mL/h)
Chicken	5	1398.4 ± 169.7^{a}	$29.6 \pm 3.3^{a, b}$	97.9 ± 24.0^{a}	$4.1 \pm 1.0^{a, b}$
Pigeon	5	138.7 ± 37.9^{c}	11.5 ± 4.3^{c}	$19.9 \pm 6.6^{b, c}$	12.7 ± 2.9^{a}
Buzzard	3	$254.1 \pm 198.9^{b, c}$	$18.4 \pm 4.1^{b, c}$	11.8 ± 5.4^{c}	17.8 ± 10.0^{a}
Eagle	4	1342.3 ± 276.1^{a}	$39.7 \pm 9.0^{a, b}$	27.8 ± 7.1^{b}	$3.5 \pm 0.6^{a, b}$
Kite	8	833.7 ± 355.5^{a}	55.3 ± 29.6^{a}	$18.3 \pm 3.8^{b, c}$	2.5 ± 1.3^{b}
Owl	6	415.9 ± 289.8^{b}	57.5 ± 27.5^{a}	$16.5 \pm 10.0^{b, c}$	2.3 ± 3.2^{b}

AUC = concentration-time area under the curve; C_{max} = peak plasma concentration; $T_{1/2}$ = plasma half-life; CL = clearance.
Shared letter symbols among groups indicate no significant difference and different letter symbols among groups denote significant difference ($p < 0.05$, Tukey's multiple test). Data are shown as mean ± SD.

In addition, the results of PCA are shown in Fig. 4A and B. The positive direction in the first principal component (component 1) indicates the larger values of AUC, C_{max} and $T_{1/2}$ i.e., higher absorption or slower excretion. On the other hand, negative direction in component 1 indicates larger values of CL, i.e., higher capacity of excretion. In the second principal component (component 2), the positive direction is influenced by the value of $T_{1/2}$, while the negative direction indicates the larger value of C_{max}. PCA plots of chickens are in positive direction of both components 1 and 2, whereas the plots of owls are in negative direction of both components 1 and 2. The plots of pigeons and buzzards are located close to each other in negative direction of components 1 and in positive direction of components 2. The plots of eagles and kites are in positive direction of components 1 and in negative direction of components 2.

3.2. Clotting time

Fig. 5 presents the time-profile of prothrombin time in chickens and buzzards. The tested samples had fibrinogen concentration >40 mg fibrinogen/dL plasma (range: 43-951 mg/dL). For these samples, PT values did not fluctuate significantly over the time course of the study compared to the PT value at 0 h (before administration) in these species.

3.3. CYP genes expression level

Fig. 6 shows the fragments per kilobase of exon per million mapped reads (FPKM) of CYP genes in Ogasawara's buzzard liver. CYP3A37 was mainly expressed followed by CYP2AC1, CYP1A4/5 and CYP2C23.

3.4. VKOR activity inhibition test

The warfarin VKOR activity inhibition test yielded IC_{50} estimates of 0.48 μM for chickens and 1.10 μM for pigeons (Table 2), whereas the warfarin IC_{50} in buzzard (0.30 μM) and Ogasawara's buzzard (0.31 μM) were relatively similar (Table 2, Fig. S7). Diphacinone VKOR activity inhibition test yielded an IC_{50} estimate of 0.47 μM for chickens, which was quite similar to that for warfarin (0.48 μM). However, the diphacinone IC_{50} in pigeons (0.31 μM) was lower than that observed for warfarin (1.10 μM) (Table 2, Fig. S8). The diphacinone IC_{50} for Blakiston's fish owl was 0.97 μM (Table 2, Fig. S9).

4. Discussion

4.1. Pharmacokinetic/pharmacodynamic of warfarin in birds

In the present study, the warfarin dose (4 mg/kg body weight) administered orally to chickens, pigeons, and buzzards was only 0.42% of the LD_{50} value in chickens (942 mg/kg) (Erickson and Urban, 2004). This exposure dose did not cause overt signs of intoxication in our study. There was no evidence of prolonged clotting time, an indicator of AR exposure, in chickens and buzzards after warfarin administration. On the other hand, a similar dose (4 mg/kg) or a lower dose (1 mg/kg) of warfarin caused prolongation of PT in rats (Chu et al., 2011). This result indicates that chickens and buzzards may have lower sensitivity to warfarin than rats.

Among studied birds, chickens had the greatest C_{max} and maintained warfarin concentration at high levels after 72 h of administration, whereas pigeons and raptors had almost no detectable warfarin in plasma by 72 h after post-administration and lower C_{max}, indicating that chickens may have a higher absorption capacity and/or a lower

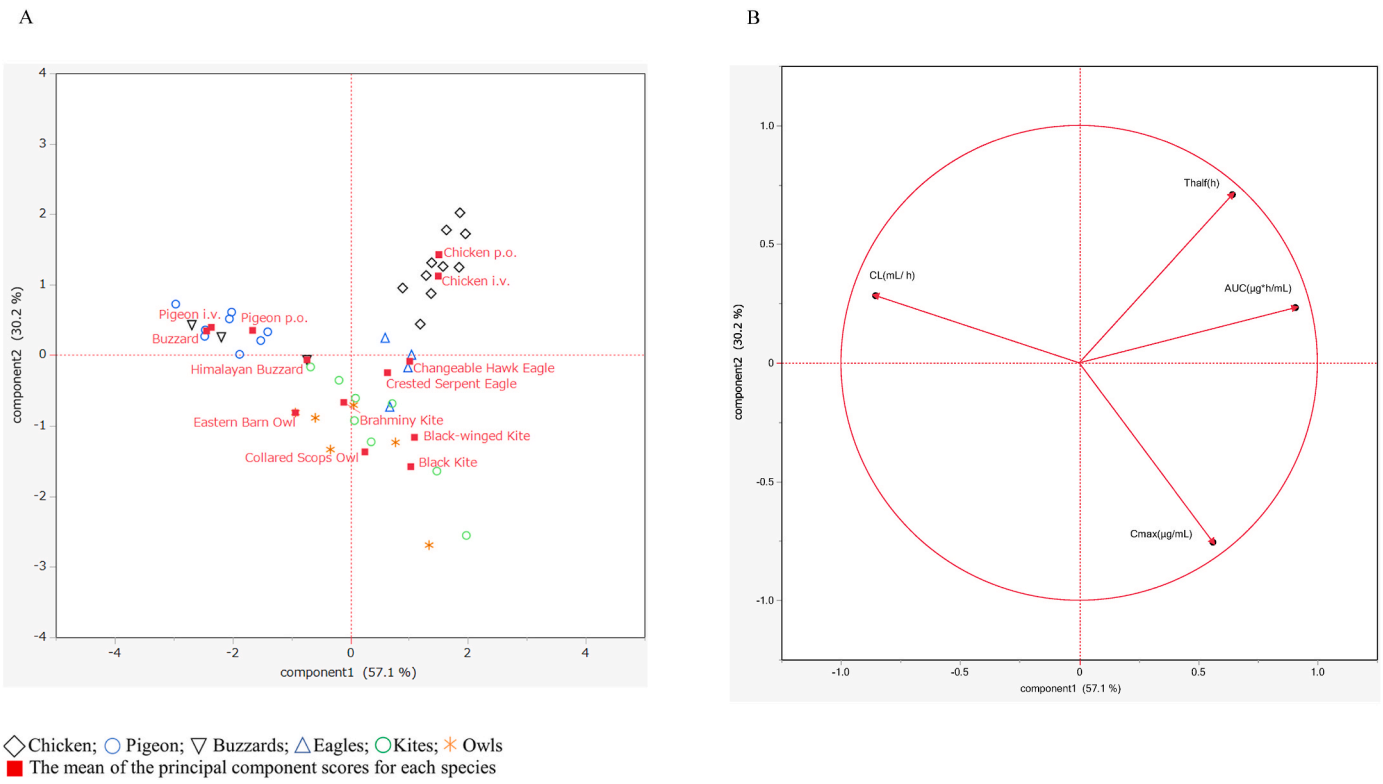

Fig. 4. A and B Principal Component Analysis (PCA) on pharmacokinetic parameters, The score plots are depicted for the principal component analysis of 4 pharmacokinetic parameters; AUC = concentration-time area under the curve; C_{max} = peak plasma concentration; $T_{1/2}$ = plasma half-life; CL = clearance <A>. Each loading vector indicates these parameters**, and each dot represents principal component score of individual <A>. The dots are colored and shaped for each related species.**

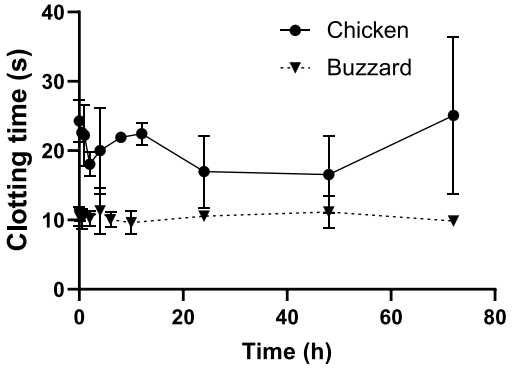

Fig. 5. Profile of prothrombin time Prothrombin time after oral administration of 4 mg/kg warfarin in chickens and buzzards. Data are shown as mean ± SD.

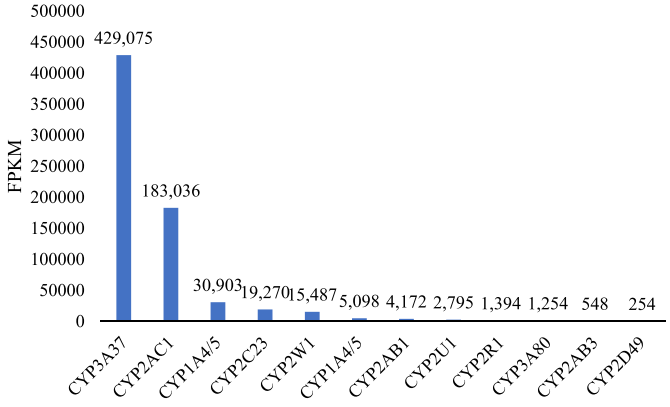

Fig. 6. Expression levels of cytochrome P450 (CYP) genes in liver of buzzard CYP gene inferred expression levels of buzzard (*Buteo japonicus toyoshimai*, n = 1) by RNA-seq. The values of Y-axis represent fragments per kilobase of exon per million mapped reads (FPKM).

metabolic capacity than other birds evaluated, however there are factors that may have influenced C_{max}. These findings contrast results of previous *in vitro* studies demonstrating that chicken and crow had a higher enzymatic efficiency of warfarin metabolism than ostrich, mallard and owl (Watanabe et al., 2015). Although the differences between *in vivo* and *in vitro* could be the major cause, the longer half-life of warfarin in chickens compared to pigeons may have been one of the factors that sustained high blood levels of warfarin in chickens. In addition, the greater concentrations of warfarin in the liver of chickens compared to those found in pigeons indicate that whole body warfarin concentration was likely higher in chickens than in pigeons.

Interspecies differences in pharmacokinetic parameters were found among avian species in this study. Notably, the half-life of warfarin in chickens is relatively long in comparison to the other avian species we studied. In addition, PCA plots showed a clear separation of chickens and other birds by $T_{1/2}$, indicating some pharmacokinetic parameters in chickens tend to be different from those of other species. This indicates that the chicken is not suitable for toxicity assessment as a model animal for many avian species. The plots of pigeons and buzzards are located close to each other, indicating that these two species show similar features on the aspect of pharmacokinetics. Although there is no difference in pharmacokinetic parameters for warfarin (i.e., AUC, C_{max}, $T_{1/2}$ and CL) among these groups of birds (especially eagles and kites), individual raptor species (e.g., eagles, kites, and owls) seemed to show a different trend in PCA plots. This tendency may help the risk assessment of target raptors on the effects of AR application in the future by evaluating the sensitivity of closely related species. In addition, prolongation of PT was

Table 2
Inhibition of Vitamin K 2,3-epoxide reductase (VKOR) activity by warfarin and diphacinone.

Species	Chicken	Pigeon	Buzzard	Ogasawara's buzzard	Blakiston's fish owl	Rat
(Scientific name)	*Gallus gallus*	*Columba livia*	*Buteo japonicus*	*Buteo japonicus toyoshimai*	*Ketupa blakistoni*	*Rattus norvegicus*
Warfarin						
V_0 (pmol/min/mg protein)	0.78	0.44	0.74	0.49	–	5.9[a]
IC_{50} (μM)	0.48	1.10	0.30	0.31	–	0.26[a]
Ki (μM)	11.3[b]	–	–	–	–	0.28[b]
Diphacinone						
V_0 (pmol/min/mg protein)	0.53	0.82	–	–	0.37	4.7[a]
IC_{50} (μM)	0.47	0.31	–	–	0.97	0.065[a]

a: data from Takeda et al., 2022), b: data from Watanabe et al., 2010).
V_0 indicates activity in the absence of warfarin or diphacinone, and IC_{50} indicates half maximal (50%) inhibitory concentration.

not observed in buzzards, however, we did not measure clotting time in other raptors. Furthermore, eagles, kites and owls showed different behavior of pharmacokinetic parameters compared to buzzards. Thus, the possibility that blood coagulation was prolonged in these species cannot be ruled out.

In contrast to rats and other mammals, 4′-hydroxy warfarin was detected as the dominant metabolite in all the avian species used in this study. This difference may be due to CYP isoforms involved in the hydroxylation of warfarin. In humans, it is known that CYP isoforms involved in warfarin metabolism include CYP2C9, CYP2C19, CYP2C8, CYP2C18, CYP1A2, and CYP3A4, and among these isoforms, CYP2C9 is likely to be the major isoform (Sun et al., 2017). *S* isomer of warfarin is metabolized primarily by CYP2C9 to 7-hydroxy warfarin, whereas *R* isomer of warfarin is metabolized primarily by CYP1A2 and CYP2A19 to 6- and 8-hydroxy warfarin, and by CYP3A4 to 10-hydroxy warfarin (Kaminsky & Zhi-Yi Zhang, 1997). In rats, CYP1A, CYP2B, CYP2C, and CYP3A isoforms are known to metabolize warfarin to 6- and 8-, 4′-, 7- and 10-hydroxy warfarin, respectively (Guengerich et al., 1982). Our previous report (Watanabe et al., 2015) indicated that CYP2C isoforms are major contributors to the metabolism of warfarin to 4′-hydroxy warfarin in birds, and *in vitro* enzymatic activity in chicken was most effective for 4′-hydroxy warfarin, followed by 6-, 7-, 8- and 10- hydroxy warfarin. These results are consistent with findings of the present study. In pigeons, our study demonstrated that CYP2C23 and CYP3A37 showed relatively higher expression levels, although expression levels of these genes varied 5-10-fold among individuals (data not shown). These two CYP isoforms seem to be involved in warfarin metabolism into 4′- and 10-hydroxy warfarin, which may explain the observed residues of 4′- and 10-hydroxy warfarin in the liver of pigeons. RNA-Seq from the liver of Ogasawara's buzzard elucidated that CYP3A37 and CYP2AC1 are mainly expressed, and it indicates that these CYP isoforms play an important role in metabolism of warfarin in buzzard. In addition, low hepatic mRNA expression of CYP3A37 and the longer half-life of warfarin (97.9 ± 24.0 h) were found in the chicken compared to predatory bird species (Watanabe et al., 2013). Based on all of these findings, we assume that the metabolism by CYP3A37 is one of the factors leading to relatively rapid metabolism of warfarin in these species.

4.2. *In vitro* effects

In addition to the metabolism of warfarin, the inhibition of VKOR activity is another factor that determines sensitivity to warfarin. For example, hepatic microsomes from warfarin resistant rats, which have mutated VKOR, are less sensitive to inhibition of enzyme activity by other FGARs and SGARs compared to warfarin sensitive rats (Lasseur et al., 2007). In the present study, IC_{50} for warfarin in chickens and pigeons were 0.48 μM and 1.10 μM, respectively, which are relatively higher than the IC_{50} in rats of 0.26 μM (data in our previous study). This indicates that VKOR activity in these species was not markedly inhibited by warfarin, and thus VKOR maintained Vitamin K at adequate concentration to sustain normal blood clotting time in chickens. Unfortunately, PT values for pigeons were not determined due to insufficient sample volume and/or inadequate fibrinogen concentration. However, it is expected that PT would not be prolonged in pigeons considering that VKOR activity may be maintained after warfarin administration as in chickens and warfarin was excreted from plasma by 72 h.

On the other hand, buzzards showed relatively lower IC_{50} for warfarin of 0.30 μM (Fig. S7, Table 2), close to the values of rats (0.26 μM). Thus, there is the possibility that the low dependence on VKOR in the Vitamin K cycle in buzzards may have prevented prolongation of coagulation by warfarin, even though VKOR is easily inhibited by warfarin. In vertebrates, VKOR activity is supported by both VKORC1 and VKORC1-like1 (VKORC1L1), a paralog of VKORC1; VKORC1L1 is less sensitive to warfarin than VKORC1 (Oldenburg et al., 2015). Nakayama et al. (2020) suggested that the expression ratio of these two genes could cause interspecies differences in avian VKOR activity. K Vitamins are divided into two major types, which are Vitamin K1 (phylloquinone) and Vitamin K2 (menaquinones, MK). Phylloquinone is synthesized in plants and is the major dietary source of Vitamin K, whereas MK has many subtypes. MK4, one of the MK subtypes, is produced from phylloquinone and is the primary physiological Vitamin K form. Both phylloquinone and MK4 can be obtained from prey including rodents (Okano et al., 2008). Raptors are predicted to intake Vitamin K from rodents or other animals that they prey on (Nakayama et al., 2020). In the present study, buzzards consumed mice during the experiment, so mice might be the source of Vitamin K.

In this study, Blakiston's fish owl had an IC_{50} for diphacinone of 0.97 μM, compared to 0.065 μM in rats. A previous study has shown that the VKOR activity of owls (snowy owl and great horned owl) is lower and more readily inhibited by warfarin than observed in chickens and turkeys (Nakayama et al., 2020). These data indicate that there may be a significant difference in the sensitivity of VKOR even among species of owls. Therefore, when ARs are applied to eradicate rodents, great care should be taken when extrapolating experimental data from different species to others for risk assessment.

Despite raptors seeming to have relatively lower sensitivity to warfarin by a single administration, it should be noted that multiple feedings on FGAR baits (e.g., warfarin, diphacinone) are required to kill target rodents (Rattner et al., 2014; Vyas and Rattner, 2012). Likewise, multiple FGAR exposures are needed to adversely non-target scavenging and raptorial birds (e.g., Rattner and Mastrota, 2018). Single oral dose exposure studies have demonstrated that SGARs (e.g., brodifacoum) are more toxic than FGAR compounds, although the difference in potency is diminished when FGARs are consumed multiple times (Rattner and Mastrota, 2018). Since multiple ARs are often detected in the liver of individual raptors (Christensen et al., 2012; Hughes et al., 2013;Lohr, 2018), it is the repeated ingestion of AR-contaminated prey that likely causes adverse effects in raptors. Therefore, it is necessary to assume that multiple AR exposures reflect the situation of routine ARs in practical use.

5. Conclusions

AR sensitivity among avian species was evaluated by *in vivo* warfarin

pharmacokinetics, *in vitro* inhibition assays of the AR target enzyme VKOR and CYPs involved in AR metabolism. Interspecific differences in AR metabolism among birds are likely due to differential expression of CYP enzymes involved in the metabolism of ARs and variation of VKOR activities among these avian species, and in particular raptors. While ecological risk assessment and mitigation efforts for ARs have been extensive, AR exposure and adverse effects in predatory and scavenging wildlife continues. Toxicokinetic and toxicodynamic data will likely assist in such risk assessments and mitigation efforts.

Author contributions statement

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envpol.2023.122837.

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