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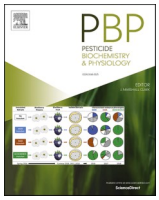

Sensitivity assessment of diphacinone by pharmacokinetic analysis in invasive black rats in the Bonin (Ogasawara) Archipelago, Japan

Kazuki Takeda [a,b,c,1,*], Keita Shimizu [a,1], Miyu Sato [a], Masafumi Katayama [d], Shouta M. M. Nakayama [c,e], Kotaro Tanaka [a], Yoshinori Ikenaka [c,f,g,h], Takuma Hashimoto [i], Ryuichi Minato [i], Yusuke Oyamada [i], Kazuyuki D. Tanaka [j], Goro Kimura [j], Tsutomu Tanikawa [j,k], Keisuke Kato [l], Taichi Kusakabe [l], Mayumi Ishizuka [c], Ryo Kamata [a]

[a] Laboratory of Toxicology, School of Veterinary Medicine, Kitasato University, E23-35-1, Towada, Aomori 034-0021, Japan
[b] Department of Computer Science, Tokyo Institute of Technology, 4259-J3-1818, Nagatsuta-cho, Midori-ku, Yokohama-shi, Kanagawa 226-0026, Japan
[c] Laboratory of Toxicology, Department of Environmental Veterinary Sciences, Graduate Faculty of Veterinary Medicine, Hokkaido University, N18 W9, Kita-ku, Sapporo 060-0818, Japan
[d] Biodiversity Division, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, Ibaraki 305-8506, Japan
[e] School of Veterinary Medicine, The University of Zambia, Great East Road, PO Box 32379, Lusaka, Zambia
[f] Water Research Group, Unit for Environmental Sciences and Management, North-West University, 11 Hoffman Street, Potchefstroom 2531, South Africa
[g] Translational Research Unit, Veterinary Teaching Hospital, Faculty of Veterinary Medicine, Hokkaido University, Sapporo, Japan
[h] One Health Research Center, Hokkaido University, Sapporo, Japan
[i] Japan Wildlife Research Center, Kotobashi 3-3-7, Sumida-ku, Tokyo 130-8606, Japan
[j] Technical Research Laboratory, Ikari Shodoku Co., Ltd, Akanehama 1-12-3, Shin-Narashino, Chiba 275-0024, Japan
[k] Animal Health Technology Department, Yamazaki Professional Collage of Animal Health Technology, Shoto2-3-10, Shibuya-Ku, Tokyo 150-0046, Japan
[l] Faculty of Pharmaceutical Sciences, Toho University, 2-2-1 Miyama, Funabashi, Chiba 274-8510, Japan

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ABSTRACT

The Bonin Archipelago is a United Nations Educational, Scientific and Cultural Organization's World Natural Heritage Site in Japan with a unique ecosystem; however, the invasive rodents preying on endemic species have been a significant concern. The anticoagulant rodenticide, diphacinone, sprayed by the Ministry of the Environment, has succeeded; however, its repeated use leads to rodenticide resistance. This study evaluated the sensitivity by *in vivo* pharmacokinetics/pharmacodynamics (PK/PD) analysis and physiologically-based pharmacokinetic modeling to diphacinone in black rats (*Rattus rattus*) captured on the Bonin Archipelago in February 2022. The Bonin rats exhibited prolonged coagulation time after diphacinone administration. They recovered earlier than susceptible black rats, indicating that Bonin rats were less susceptible, though there were no genetic mutations in *Vkorc1*, the target enzyme of diphacinone. After the administration of diphacinone, hepatic expression levels of *Fsp1*, identified as the vitamin K reductase, was decreased, however, the Bonin rats exhibited the most minor suppression. The PK analysis showed that the excretion capacity of the Bonin rats was lower than that of the resistant black rats. In the PBPK modeling, the resistant black rats showed higher clearance than the Bonin and susceptible black rats due to high hepatic metabolic capacity. The Bonin rats demonstrated slow absorption and relatively low clearance. This study highlighted the reduced rodenticide-sensitive tendency of wild black rats in the Bonin Archipelago at an *in vivo* phenotype level. At the same time, they do not have known rodenticide resistance mechanisms, such as hepatic metabolic enhancement or *Vkorc1* mutations. It is crucial to monitor the biological levels to evaluate rodenticide sensitivity accurately.

Abbreviations: AAALAC, Association for Assessment and Accreditation of Laboratory Animal Care; AIFM2, Apoptosis inducing factor mitochondria associated 2; AUC, Area under curve; Cl, Clearance; C_{max}, Maximum plasma concentration; CYP, Cytochrome P450; FSP1, Ferroptosis suppressor protein 1; LCMS, Liquid Chromatograph - Mass Spectrometry; LOD, Limit of detection; LOQ, Limit of quantification; MRT, Mean residence time; NCA, Non-compartment analysis; PBPK, Physiologically-based pharmacokinetics; PD, Pharmacodynamics; PK, Pharmacokinetics; PT-INR, Prothrombin time international normalized ratio; R-rat, Rodenticide-resistant black rat; SD rat, Sprague-Dawley rat; S-rat, Rodenticide-susceptible black rat; $T_{1/2}$, Terminal half-life; T_{max}, Time to reach C_{max}; UNESCO, United Nations Educational, Scientific and Cultural Organization; V_c, Volume of the central compartment; VKORC1, Vitamin K epoxide reductase complex subunit 1.

* Corresponding author at: Laboratory of Toxicology, School of Veterinary Medicine, Kitasato University, E23-35-1, Towada, Aomori 034-0021, Japan.
E-mail address: takeda@vmas.kitasato-u.ac.jp (K. Takeda).

[1] Equal contribution, thus they will put their name first on their own CV.

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1. Introduction

The Bonin (Ogasawara) Archipelago is located about 1000 km south of Tokyo. It has a unique ecosystem represented by endemic species, such as the Bonin flying fox (*Pteropus pselaphon*) and the red-headed wood pigeon (*Columba janthina nitens*) (Kawakami, 2010). The flora comprises 260 flowering plant species, of which 112 are endemic (Toyoda, 2014). The native terrestrial fauna is limited to one fruit bat, one lizard, 15 land birds, 800 insects, and 100 land snails (Shimizu, 2003). The archipelago was inscribed on World Heritage List of United Nations Educational, Scientific and Cultural Organization (UNESCO) in 2011, and the rarity of this ecosystem is internationally recognized. However, ecosystem disturbance by invasive species has been a concern, particularly the high reproductive and invasive impact of the brown rat (*Rattus norvegicus*) and black rat (*Rattus rattus*). The damage to endemic vegetation, such as the takono-ki (*Pandanus boninensis*), and predation of small seabirds, such as the Bulwer's petrel (*Bulweria bulwerii*), have been confirmed, and countermeasures are essential (Abe, 2007; Yabe et al., 2009). Several applications of diphacinone-containing rodenticides, led by the Japanese Ministry of the Environment, have been performed (Hashimoto, 2010). Diphacinone is the only rodenticide approved by the Ministry of the Environment for use in the Bonin Islands due to its relatively low expected impact on wildlife.

The mechanism of action of anticoagulant rodenticides, including diphacinone, involves hemorrhagic toxicity caused by inhibiting blood coagulation (Rattner et al., 2014). The blood clotting factors II, VII, IX, and X are modified by vitamin K-dependent gamma-carboxylation to acquire coagulation activity (Cain et al., 1998). The activated form of vitamin K (hydroquinone) that is used in this reaction is converted to an inactive epoxide form, which is then reactivated by vitamin K epoxide reductase complex subunit 1 (VKORC1) (vitamin K cycle) (Oldenburg et al., 2008). The anticoagulant rodenticides compete with VKORC1 by binding to its active site, depleting vitamin K-dependent clotting factors (Czogalla et al., 2017; Rost et al., 2004).

The repeated use of rodenticides has led to the emergence of populations that exhibit resistance in various parts of the world and are known as super rats, making it challenging to control them (Rost et al., 2004, 2009). The super rats have been found in Japan, where 80% of the captured rats in the Shinjuku ward, Tokyo, were resistant, indicating their risk of emergence (Tanikawa and Suzuki, 1993). The reported mechanisms of rodenticide resistance can be broadly divided into two categories. One is a genetic mutation in the target molecule, VKORC1, of rodenticides, which decreases the binding affinity of VKOR and rodenticides, resulting in resistance (Rost et al., 2004). The other is the improvement in the detoxification and metabolism ability of rodenticides by the drug-metabolizing enzyme, cytochrome P450 (CYP), leading to the rapid excretion of rodenticides from the body, resulting in the development of resistance (Horak et al., 2015; Ishizuka et al., 2007; Markussen et al., 2007). Several super rats found worldwide developed resistance due to the former mechanism (Buckle and Smith, 2015; Rost et al., 2009). The genetic sequencing of VKOR and quantification of CYP gene expression can be scientific evidence for evaluating rodenticide resistance. However, since rodenticide resistance without *Vkorc1* mutation and high warfarin excretion capacity without enhanced CYP expression have been observed in some populations; therefore, *in vivo* susceptibility evaluation is essential (Heiberg, 2009; Takeda et al., 2016).

Effective rodenticide dispersal operations are essential for the continued success of pest control efforts in the Bonin Islands. However, to date, the analysis of rodenticide resistance in invasive black rats inhabiting the Bonin Islands has been limited to the sequencing of *Vkorc1* in resistant individuals to confirm mutations, and no analysis at the biological level has been conducted. In this study, the largest black rats on Chichijima Island were captured and diphacinone was administered to evaluate the pharmacodynamic and pharmacokinetic responses. They quantitatively analyzed the gene expression levels of the target molecule, VKOR, and drug metabolism enzyme, CYP, in their liver samples using quantitative PCR (qPCR). The widely used Sprague–Dawley (SD) rats and closed colonies of rodenticide-susceptible and -resistant black rats were used for comparative analysis. Additionally, to examine the factors of inter- and intra-species/strains differences in pharmacokinetics, we reproduced the PK dynamics by physiological-based pharmacokinetic (PBPK) modeling.

2. Materials & methods

2.1. Materials

Syringes (1 mL) and butterfly needles (27 G) were obtained from Terumo Co. (Tokyo, Japan). The administration needles were procured from Primetech Co. (Tokyo, Japan). Diphacinone, ethanol, methanol, acetone, acetonitrile, isoflurane, PEG400, DDW, formic acid, sodium acetate, and trisodium citrate were purchased from FUJIFILM Wako Pure Chemical Co. (Osaka, Japan). The TRI reagent was obtained from the Molecular Research Center, Inc. (Cincinnati, OH). Diphacinone-d4 (indanedione-4, 5, 6, 7-d4) was obtained from C/D/N Isotopes Inc. (Quebec, Canada). The glass vials for liquid chromatography and anhydrous magnesium sulfate powder (Final Drying Pouches-$MgSO_4$) were purchased from Agilent Technologies Ltd. (Santa Clara, CA). Monohydroxy diphacinone was synthesized at Toho University.

2.2. Animals

The Jcl: SD (SD rats) and three strains of black rats (*Rattus rattus*) were used in this study. Six nine-week-old SD rats were purchased from Nippon Clare Corporation (Tokyo) and acclimated for one week before being treated at ten weeks of age. The light-dark cycle was maintained at 12 h day and night, and the rats were fed *ad libitum* (solid feed CE-2, Nippon Crea Co.). The closed colonies of rodenticide-susceptible and -resistant black rats were supplied by IKARI SHODOKU Co., Ltd. (Tokyo, Japan). These two strains were caught in the wild and maintained as closed colonies in their laboratory for over 20 years (Takeda et al., 2016). The susceptible rats (S-rats) originated from Chichijima Island before the rodenticide spread began, while the resistant rats (R-rats) were captured from Shinjuku, Tokyo, Japan. The R-rats had a genetic mutation in *Vkorc1* (Leu76Pro) (Takeda et al., 2016). The body weight of S-rats was 134.3 ± 4.0 g and were an estimated 16.6 ± 4.6-weeks-old, and the body weight of R-rats was 128.6 ± 11.7 g and 10.7 ± 1.1 weeks old (mean $\pm$ SD, $N = 5$, males, each). The above-week age was estimated from the dry weight of the eye lens according to Tanikawa's method (Tanikawa, 1993). Bonin wild black rats were captured with a cage trap in the Omura district of Chichijima in February 2022 (Fig. 1). All captured rats were identified as black rats based on their physical appearance, and only an influx of black rats has been confirmed on Chichijima. Their average body weight was 139.4 ± 22.1 g, and their average estimated age was 10.6 ± 3.8 weeks ($N = 7$, 4 males and 3 females). Information on each captured black rat is shown in Table 1.

2.3. Oral administration test of diphacinone

Diphacinone (0.5 mg/kg of body weight) was orally administered to the rodents. It was first dissolved in acetone and then in PEG400: purified water (1:1) solution at a final concentration of 0.5 mg/mL. The final concentration of acetone in the solution was 5%. The dose was set at 0.5 mg/kg to allow the completion of 96 h of observation without any lethality based on a preliminary study using SD rats. The rodents were fasted for 12 h prior to administration. Approximately 50 μL of blood were collected from the tail vein at 0.16, 1, 2, 4, 6, 10, 17, 24, 30, 48, 72, and 96 h after the administration. Prothrombin time (international normalized ratio: PT-INR (Poller, 2004)) was measured directly from an aliquot (10 μL) of whole blood immediately after collecting the samples at 0.16, 10, 17, 24, 30, 48, 72, and 96 h using CoaguCheck XS (Roche

Diagnostics, Basel, Switzerland). The plasma was isolated from the rest of the blood with 3.2% of sodium citrate by centrifugation. The rats were euthanized by isoflurane overdose after blood sampling. Immediately after, the rats were laparotomized, and small pieces of the liver and duodenum were collected for following genetic analysis. These samples were immersed in RNAlater (Thermo Fisher Scientific, Inc. Waltham, MA) and stored at −80 °C until use.

All animal care and experimental procedures were performed following the Guidelines of the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC International) and approved by the Animal Care and Use Committee of the Faculty of Veterinary Medicine, Hokkaido University (Approved number; 21–0120) and the Institutional Animal Care and Use Committee of School of Veterinary Medicine, Kitasato University (No. 20–107).

Table 1
Individual information.

ID	Sex	Body weight (g)	Estimated age (weeks)
A	F	120.7	19.1
B	F	107.7	6.3
C	M	137.3	7.5
D	M	164.5	8.3
E	M	139.5	9.8
F	F	174.5	11.1
G	M	126.3	9.2

The information on wild black rats captured on Chichijima Island was used in this study. The body weights were measured immediately before diphacinone administration. The weekly age was estimated from the dry weight of their eye lens. The pharmacokinetics/pharmacodynamics kinetics for each individual are shown in the supplementary materials.

2.4. Diphacinone extraction and LCMS measurement

The extraction of diphacinone and monohydroxy diphacinone (OH-diphacinone) from the plasma was performed based on the QuEChERS method (Quick, Easy, Cheap, Effective, Rugged, and Safe) (Schenck and Hobbs, 2004). Around 5 μL of plasma, 475 μL of 0.1 M sodium acetate buffer (pH 5.0), and 20 μL of 1 μM diphacinone-d4 were dissolved in acetonitrile containing 1% formic acid, transferred to 2.0 mL plastic tubes, and mixed with the Micro Smash MS-100R (Tomy Seiko Co., Ltd., Tokyo, Japan) at 2000 rpm for 5 min. Then, 0.35 g of anhydrous magnesium sulfate powder was added for dehydration and mixed in the same manner for 5 min. 100 μL of the supernatant (acetonitrile phase) was transferred to a glass vial for liquid chromatography with an insert and diluted by adding 100 μL of 0.1% formic acid. The concentrations of diphacinone and OH-diphacinone were determined by LC/MS/MS (6495 triple quad LC/MS; Agilent Technologies, Santa Clara, CA) equipped with SM-C18 column (3.0 × 100 mm, φ3 μm, Imtact, Kyoto, Japan). The mobile phase A comprised 0.05% acetic acid and 10 mM ammonium acetate in DDW. The mobile phase B comprised 10 mM of ammonium acetate in acetonitrile. The applied gradient was: $t = 0$–0.2 min, 10% solvent B; $t = 6.0$ min, 90% solvent B; $t = 6.01$–7.5 min, 100% solvent B; $t = 7.51$–9.0 min, 100% solvent B. Injection volume, flow rate, and column temperature were 10 μL, 0.8 mL/min, and 45 °C, respectively. The collision energies (CE) and other MS parameters were optimized and shown in Table 2.

2.5. Real-time PCR

The expression levels of *Vkorc1*, vitamin K epoxide reductase, a target enzyme of anticoagulant rodenticide, and *Aifm2*, which encodes ferroptosis suppressor protein 1, identified vitamin K quinone reductase (Mishima et al., 2022) and the drug-metabolizing enzyme cytochrome P450, can contribute to rodenticide resistance and is measured by quantitative PCR (qPCR) according to a previous report (Kamata et al., 2022). Briefly, the total RNA was extracted from the liver and duodenum of the rodents using TRI reagent and Monarch RNA Purification Columns (New England BioLabs, Ipswich, MA). Reverse transcription from mRNA was performed using the LunaScript® RT SuperMix Kit with standard protocols (New England BioLabs). The cDNA was amplified using Luna® Universal qPCR Master Mix and corresponding primers for qPCR. The primers were designed using the National Center for Biotechnology Information Primer designing tool (Ye et al., 2012). The primer information is shown in Table S1. The PCR conditions were as follows: initial denaturation of 95 °C for 60 s, 40 cycles of 95 °C for 15 s, and 60 °C for 30 s. Real-time PCR was conducted for each sample using the StepOnePlus™ Real-Time PCR System (Thermo Fisher Scientific, Inc.). β-actin (*Actb*) was used as an internal control, and the fold change was calculated using the $2^{-\Delta\Delta Ct}$ method.

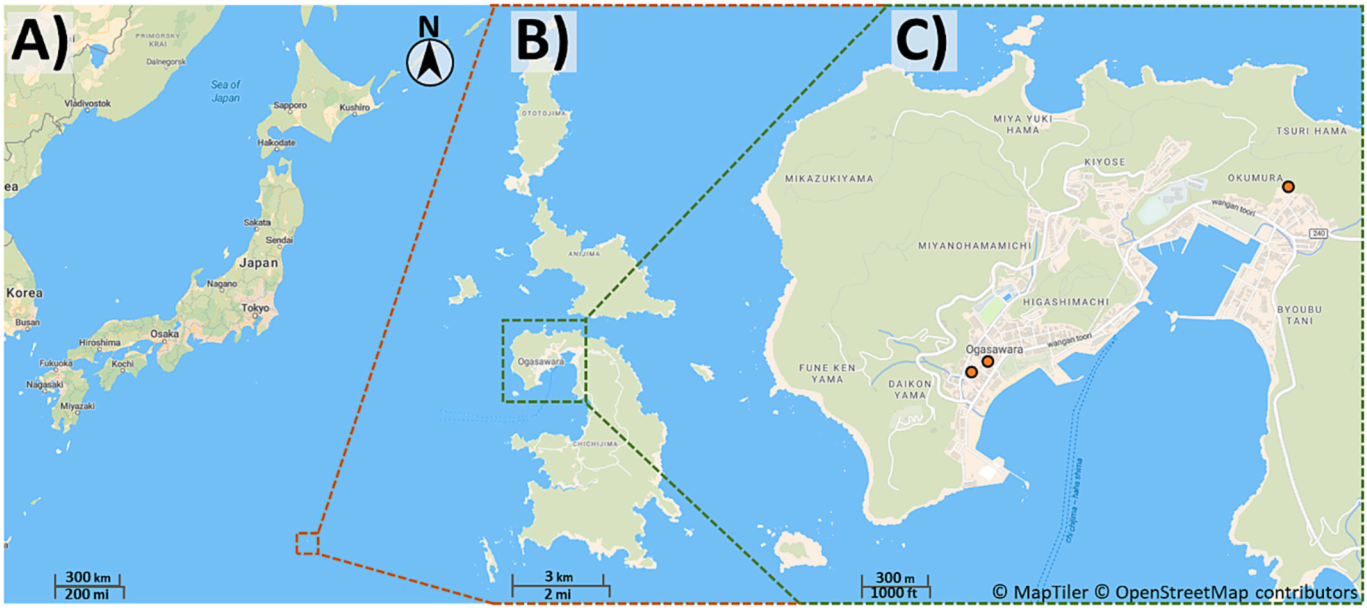

Fig. 1. A) The location of the Bonin Archipelago, which is located about 1000 km from the Japanese mainland. B) The overall view of Chichijima Island. Chichijima is one of the two inhabited islands in the Archipelago, where 80% of the total population lives. C) The sampling site on Chichijima. The cage trap was placed at the location indicated by the orange circle after obtaining permission from the Chichijima Village Office.

Table 2
Method validation of quantification by liquid chromatography-mass spectrometry.

	Recovery rate (%)	LOD (nM)	LOQ (nM)	LOD (ng/mL)	LOQ (ng/mL)	Ionization mode	Precursor (m/z)	Product (m/z)	Dwell time (ms)	CE (V)
Diphacinone	102.0 ± 6.8	7.1	21.5	2.4	7.3	negative	339	116	50	48
Monohydroxydiphacinone	104.9 ± 7.4	5.7	17.3	2.0	6.2	negative	355.1	183.2	50	24
Diphacinone-d4	104.8 ± 5.1	NA	NA	NA	NA	negative	343	120	50	48

The recovery rates of each compound was evaluated by the spike and recovery test with plasma (mean ± SEM, N = 4). Limit of detection (LOD) and quantification (LOQ) was calculated with eight replicates of standard curve. LOD and LOQ of diphacinone-d4 were not calculated because it was added at a final concentration of 100 nM for all samples and standards.

2.6. Sequence analysis

The gene sequences of each gene quantified by qPCR confirmed mutations, as previously reported (Takeda et al., 2016). Briefly, cDNA was amplified by conventional PCR using PrimeSTAR® Max DNA Polymerase (Takara Bio Inc., Shiga, Japan). The PCR profile consisted of 10 s at 98 °C, followed by 35 cycles of 10 s at 98 °C, 5 s at 60 °C, and 30 s at 72 °C. The PCR products were purified by NucleoSpin Gel and PCR Clean-up columns (MACHEREY-NAGEL, Nordrhein-Westfalen, Germany). The Sanger sequencing was contracted to Fasmac Co., Ltd. (Kanagawa, Japan).

2.7. PK analysis

The non-compartment models were fitted to the plasma concentration-time data of diphacinone and hydroxywarfarins to calculate standard pharmacokinetic parameters (Takeda et al., 2022). The fitting was performed with PKPlus 2.5 (Simulation Plus, Inc., Lancaster, CA). Terminal slope detection was automatic, and the weighting parameter was set as Unity.

2.8. PBPK modeling

The physiologically-based pharmacokinetic (PBPK) modeling was performed using GastroPlus v9.8 (Simulation Plus, Inc.) to understand each rodent's physiological characteristics based on the PK analysis results. The physical properties of diphacinone were estimated using ADMET Predictor with their default settings. Modeling was performed in a compartmental model setting, and the measured values of V_c and Cl were used for SD rats and the susceptible group. For other physiological parameters, reference values of laboratory rats with built-in GastroPlus were used. The fitting to the measured values was performed by minimizing an error function with each parameter as the objective variable using the Optimization Module of GastroPlus.

2.9. Statistical analysis

The statistical analysis was performed by JMP Pro 17 (SAS Institute Inc., Cary, NC). The Tukey–Kramer HSD test was used for multiple comparisons among the four groups of rodents. The significance level was defined as a p-value <0.05.

3. Results

3.1. Pharmacodynamics of diphacinone: prothrombin time

The prothrombin time (PT-INR) was measured to assess the effects of diphacinone administration (Fig. 2). The normal reference range for PT-INR in humans is 1.0, and the upper limit of CoaguChek®XS used in this study is 8.0. In this analysis, the measurement values above 8.0 were treated as 8.0. The PT-INR values of four rodent strains 10 min after administrations were normal at 1.0 ± 0.03 (mean ± SEM, Fig. 2A). PT-INR increased 6 h after administration in all four rodent strains, including the resistant rats (R-rats). In SD rats, diphacinone had the most pronounced effect, maintaining the upper limit value of 8.0 between 24 and 58 h after administration. Alternatively, the prolonged coagulation time in the S-rats began to recover soon after the upper limit of 8 at 32 h and recovered to the near reference range of 1.6 ± 0.3 at 96 h. In the R-rats, the maximum value rose only to 4.4 ± 1.3 at 24 h after administration. The Bonin rats had a normal PT-INR of 1.0 ± 0.06 immediately after administration. After reaching a maximum value of 6.4 ± 0.4 at 32 h after administration, they showed a relatively rapid recovery trend and recovered to 1.6 ± 0.4 at 72 h after administration, which was near the reference range. The results for each Bonin rat are shown in Fig. S1. No significant differences were found between males and females (Two-way repeated ANOVA, $p > 0.05$). To quantitatively and statistically compare the PD profile of diphacinone between the rodent strains, the area-under-curve (AUC) of the kinetics was calculated (Fig. 2B); the smaller AUC indicates the weaker effect of diphacinone. The Bonin rats showed significantly lower AUCs than SD and S-rats (Tukey–Kramer's HSD test, $p < 0.05$ for S-rats, $p < 0.001$ for SD rats).

3.2. Method validation for LCMS measurement

To verify the efficiency of extraction of diphacinone from plasma by the QuEChERS method, a spike and recovery test with a blank plasma of SD rats was performed to investigate the recovery rate ($N = 4$). The recovery rate of each compound is described in Table 2. The limit of detection (LOD) and the limit of quantification (LOQ) calculated by the standard curve ($N = 8$) are described in Table 2. The recovery rates were favorable for diphacinone, hydroxylated diphacinone, and the internal standard diphacinone-d4 were around 100%. The LODs of diphacinone and hydroxylated diphacinone were sensitive for performing PK analysis with 5 μL of plasma.

3.3. Pharmacokinetics of diphacinone and OH-diphacinone

Fig. 3A shows diphacinone plasma concentration-time profiles after oral administration at a 0.5 mg/kg dose. These were subjected to non-compartment analysis (NCA), and the pharmacokinetic parameters were calculated, as shown in Table 3. The absorption of diphacinone was relatively slow, with a T_{max} ranging from 4.0 to 10.8 h. The R-rats showed lower AUC than SD and Bonin rats, suggesting that diphacinone in the plasma was low. However, the MRT, a parameter to evaluate the excretion capacity, showed significantly longer time in SD rats than in the three black rat groups. In contrast, Bonin rats were characterized by a relatively high AUC and low excretion capacity (low Cl).

Diphacinone is hydroxylated by cytochrome P450. Monohydroxylated diphacinone (OH-diphacinone) was synthesized as a standard for LCMS measurement, and its kinetics were analyzed (Fig. 3B). The OH-diphacinone was detected from the plasma at high concentrations, and its C_{max} was almost the same as that of the parent compound (Table 3). However, the elimination of OH-diphacinone was relatively slow; diphacinone disappeared from the plasma of almost all strains at 72 h after the administration, whereas OH-diphacinone appeared to be in the elimination phase even at 96 h. PK kinetics for individual Bonin rats are also shown in Fig. S2.

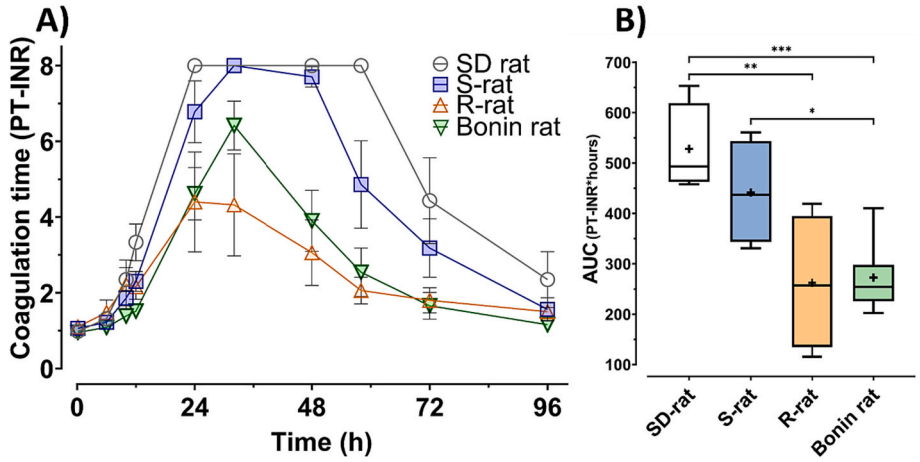

Fig. 2. A) Pharmacodynamic analysis after oral administration of diphacinone. The y-axis indicates the prothrombin time international normalized ratio (PT-INR). A high PT-INR indicates that a long time is required for blood coagulation. Normal PT-INR is approximately 1.0, and the maximum value that the CoaguCheck XS used in this study was 8.0. The open circles represent the mean values of SD rats ($n = 6$), blue squares represent those of sensitive black rats (S-rats, $n = 5$), orange triangles are of resistant black rats (R-rats, $n = 4$), and green inverted triangles represent those of wild black rats captured in Chichijima (Bonin rat, $n = 7$). B) The area-under-curve (AUC) of the pharmacodynamics. The data are shown in box-and-whisker plots with boxes indicating each quartile and median, + denotes the mean value, and the whiskers denote the maximum and minimum values. * represents significant differences detected by the Tukey–Kramer's HSD test (*; $p < 0.05$, **; $p < 0.01$, ***; $p < 0.001$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

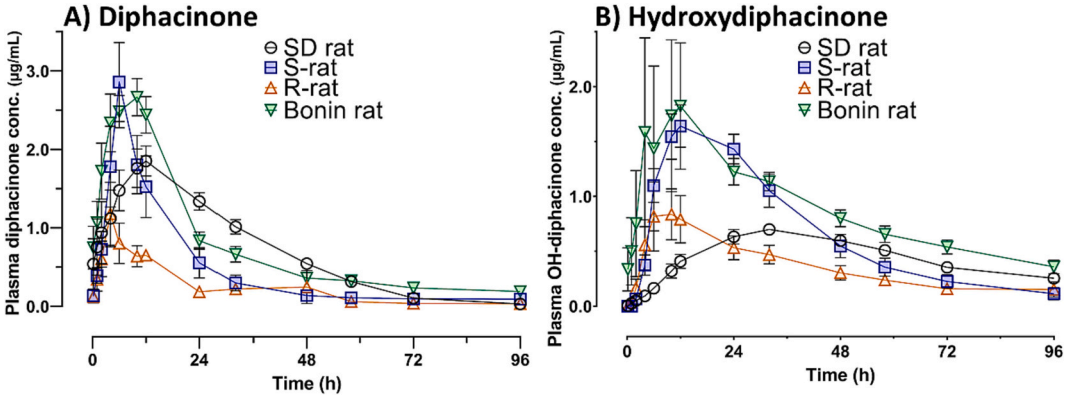

Fig. 3. Pharmacokinetic analysis of diphacinone (A) and hydroxydiphacinone (B). The blood collection was performed at 0.16, 1, 2, 4, 6, 10, 17, 24, 30, 48, 72, and 96 h after administration. Each data point represents the mean ± SEM. A non-compartmental model was applied to calculate the pharmacokinetic parameters shown in Table 3.

Table 3
Pharmacokinetics parameters.

	AUC_{last}(μg*h/mL)	C_{max} (μg/mL)	T_{max} (h)	Cl (μL/h)	MRT_{inf} (h)	$T_{1/2}$ (h)
SD rat	53.98 ± 4.75a	1.69 ± 0.13	10.8 ± 3.1	3.23 ± 0.30a	27.4 ± 1.8a	13.6 ± 0.8a
S-rat	36.46 ± 8.56ab	2.86 ± 0.50	6.0 ± 0.0	2.11 ± 0.47ab	14.4 ± 1.3b	8.5 ± 1.2b
R-rat	18.70 ± 2.69b	1.50 ± 0.40	4.0 ± 0.0	3.11 ± 0.44a	16.1 ± 3.1b	9.9 ± 2.0ab
Bonin rat	48.77 ± 4.83a	2.72 ± 0.24	8.5 ± 1.0	1.28 ± 0.18b	18.2 ± 1.3b	10.4 ± 1.0ab

The mean ± SEM of pharmacokinetic parameters obtained by non-compartmental analysis is shown. AUC_{last}, area under concentration-time curve from time 0 to 96 h; C_{max}, maximum plasma concentration; T_{max}, time to reach C_{max}; Cl, apparent total clearance of the drug from the plasma after oral administration; MRT, mean residence time from time 0 to infinity; $T_{1/2}$, terminal half-life. The different letters indicate significant differences between the two (Tukey–Kramer's HSD test, $p < 0.05$).

3.4. Genetic analysis

Genetic mutations in the diphacinone target enzyme, *Vkorc1*, and polymorphisms or an increased expression of cytochrome P450 have been reported as *in vivo* factors affecting rodenticide sensitivity in rodents and humans. This study analyzed the sequences of *Vkorc1* and three *CYP* genes, *Cyp1a2* and *Cyp2c11* (male-specific), which is highly expressed in rat liver, and *Cyp3a9*, which is essential in gastrointestinal metabolism. However, sequence analysis did not detect any mutations that resulted in amino acid mutations in *Vkorc1* and *Cyp1a2*, *2c11*, or *3a9* genes in all Bonin rats (data not shown).

The relative quantification of these gene expression levels by the ΔΔCt method is shown in Fig. 4. The control group was S-rats (male, $N = 3$) that had not been treated with diphacinone. In the liver, there were significant differences only in *Cyp2c11*, with R-rat showing a significantly higher expression level of about 4-fold compared to the others; Bonin rats showed a significantly lower *Cyp2c11* expression level than S-rats. The R-rats showed significantly higher P450 expression levels in the duodenum. In addition, the hepatic expression of ferroptosis suppressor protein 1 (FSP1), also known as apoptosis inducing factor mitochondria associated 2 (*Aifm2*), identified as a vitamin K reductase,

was quantified. The R-rat showed significantly lower *Aifm2* expression than the S- and Bonin rats, but there were no significant differences between the S- and Bonin rats.

3.5. PBPK modeling

PBPK modeling was performed to characterize the pharmacokinetics of each rodent strain. Fig. 5A shows the modeling results for SD rats. The graph showed the fitting results with built-in software default values for laboratory rats (dashed line) and optimizing parameters to best fit the measured values (solid line). The changes, including clearance and V_c per body weight, were changed to the actual PK analysis measured values from estimated values by the software, and the gastric transit time was extended from the default value of 0.25 to 5 h. In addition, the enzyme activity value of CYP2C11, identified by the ADMET Predictor module as the enzyme responsible for diphacinone metabolism, was reduced as a factor of 0.1 for V_{max} and 2 for K_m. Fig. 5B showed PBPK fitting for the S-rat. Initially, V_c, Cl, gastric transit time, and CYP activity were set to the same values as of the SD rat, and body weight and dose were set to the S-rat values, which did not fit the observed pharmacokinetics. When the PK analysis measured S-rats values for V_c and Cl, and gastric transit time was reduced from 5 to 4.5 h, a good fit was obtained except for the highest concentration. Subsequent modeling of the R-rat and the Bonin rat was performed using the PBPK values of the S-rat as a reference. Fig. 5C shows the R-rat modeling results; using S-rat parameters resulted in an overall shift to the upper side of the measured values. Therefore, the metabolic capacity was increased by changing V_{max} from 0.1 to 4-fold, K_m from 2 to 0.65-fold, and the first-pass effect in the intestinal tract from 0 to 5%, which showed the best fitting in the absorption and early excretion phases. However, in the late excretory phase, the excretory capacity was much higher than measured values, meaning that almost all drugs were excreted at 30 h, and fitting for slow excretion in the late excretory phase was not obtained by optimization using the Optimization Module. Fig. 5D showed the modeling results for Bonin rats; only the slope in the absorption phase was reproduced when the S-rat parameters were appropriated. Therefore, unlike the R-rat, optimizing metabolic enzyme activity values did not affect the fitting. Therefore, the clearance per body weight was reduced by 0.03 from the value of the S-rat without changing the enzyme activity value, and the gastric transit time was changed from 4.5 to 5.5 h, resulting in the best fitting.

4. Discussion

Since rodenticide-resistant rodents have been well-documented, it is essential to conduct susceptibility assessments when utilizing rodenticides (Buckle and Smith, 2015). The Bonin Islands, which are the focus of this study, are recognized as a UNESCO World Natural Heritage site. However, they have been plagued by non-native rodents and subjected to multiple rodenticide applications. Despite this, no biological susceptibility assessment of non-native rodents has been conducted on the archipelago. PKPD analysis following a single dose and genetic analysis for rodenticide susceptibility evaluation help confirm VKOR mutation and metabolism if living organisms are available. This study performed PKPD analysis on the black rats captured in 2022.

The Chichijima wild black rats (Bonin rats) analyzed in this study exhibited normal PT-INR and diphacinone concentrations below the LOD value upon LCMS measurement performed at the onset of the experiment, indicating that they had not been previously exposed to diphacinone. The PT-INR of the Bonin rats was significantly prolonged following the administration; however, it recovered rapidly. The AUC of PD kinetics was significantly lower than that of the S-rats and SD rats, while it was not significantly different from the R-rats (Fig. 2). In other words, their response to diphacinone was more akin to that of the R-rats. However, the Bonin rats displayed nearly identical PK kinetics to S-rats; both S-rats and Bonin rats exhibited high C_{max} values, but Bonin rats excreted diphacinone more rapidly than SD rats, as indicated by MRT and $T_{1/2}$ values. The AUC was lower in the R-rats, but this may be due to a first-pass effect in the intestinal tract, resulting in a lower amount of diphacinone absorbed into the body (Fig. 3A and Table 3). This is supported by high P450 expression in the gastrointestinal tract as determined by qPCR and PBPK results (Figs. 4 and 5C). The plasma concentration of hydroxylated diphacinone was comparable to that of diphacinone, suggesting that diphacinone is readily hydroxylated (Fig. 3B). Additionally, the contribution of phase II conjugation reactions to diphacinone metabolism was assessed by comparing it with β-glucuronidase treatment during extraction. However, it did not affect the amount of diphacinone hydroxide detected; indicating that either the contribution of phase II conjugation reactions to diphacinone metabolism is low or that diphacinone is deconjugated during this extraction process (data not shown). Moreover, the substantial contribution of hydroxylation reactions suggested that CYPs played a significant role in susceptibility to diphacinone. While hydroxylated organic compounds are generally excreted more rapidly, in the case of diphacinone, hydroxylated forms exhibited lower excretion parameters, such

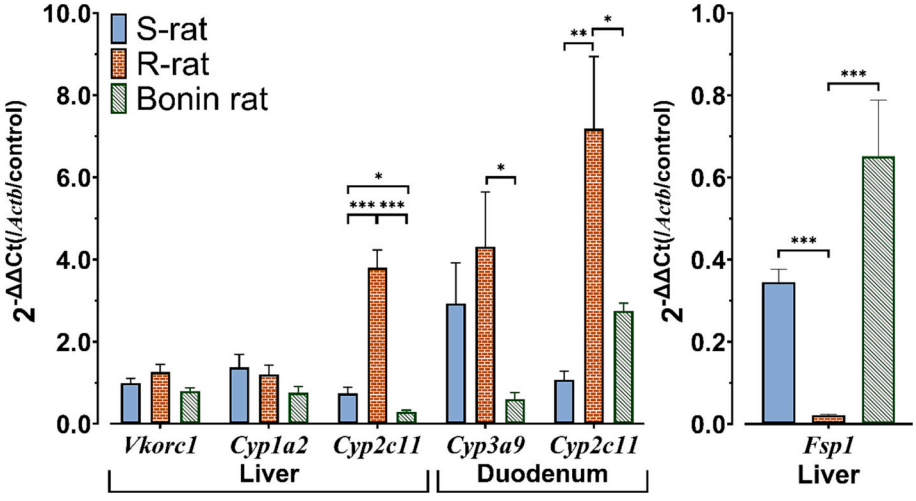

Fig. 4. Real-time quantitative polymerase chain reaction. The y-axis shows the relative values using β-actin (*Actb*) as the calibration gene. As a control group, mRNA from susceptible black rats that had not been administered diphacinone was used. The data are represented as the mean ± SEM. All data are measured in duplicate. The significant differences are indicated as * ($p < 0.05$), * *($p < 0.01$), and * ** ($p < 0.001$) detected by the Tukey–Kramer's HSD test.

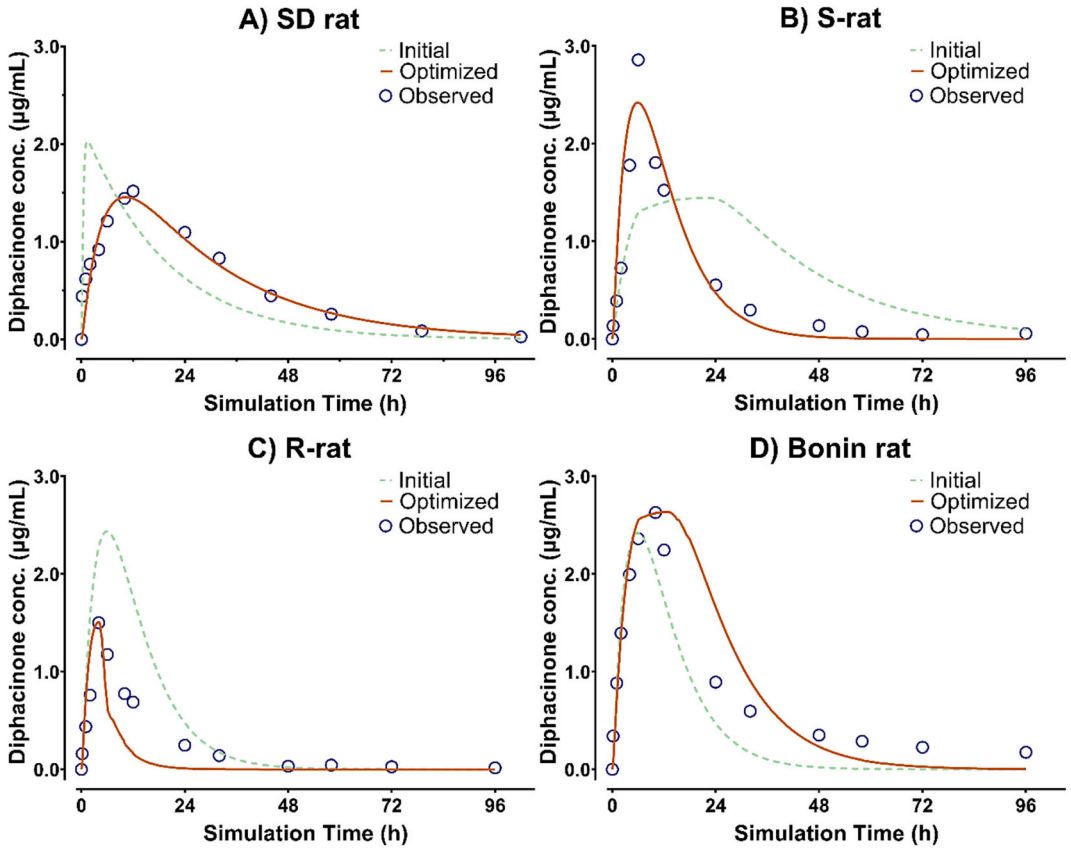

Fig. 5. Physiologically-based pharmacokinetic modeling. The dots show the observed values in Fig. 3A, the green dashed line shows the modeling results using the software's built-in default values, and the blue solid line shows the modeling results with optimization by changing parameters. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

as MRT and $T_{1/2}$, than diphacinone (Table 3). Although it is not well understood whether hydroxydiphacinone retains its ability to inhibit VKOR, some hydroxylated warfarins inhibit VKOR *in vitro* (Haque et al., 2014), indicating that further investigations into the VKOR inhibitory potential of long-term residues of hydroxydiphacinone was warranted. In this study, one particular hydroxydiphacinone was synthesized and subjected to LCMS analysis. Alternatively, warfarin has five hydroxylated forms (Guengerich et al., 1982), and diphacinone may have other hydroxylated forms that were not observed in this study. However, the high plasma concentration levels of the observed hydroxydiphacinone suggested that it is the major one (Fig. 3). One of the Bonin rats exhibited extraordinarily high levels of hydroxydiphacinone (Fig. S3); its CYPs expression was not upregulated compared to the other Bonin rats. Hence, the cause of this is unknown. There were no significant sex differences in the PK/PD parameters within the Bonin rats (Figs. S1-S2).

The PBPK analysis revealed characteristics of four rodent strains and diphacinone. PBPK modeling evaluated the pharmacokinetics based on physiological functions by simulating absorption and metabolism in each organ based on the physical properties of chemical substances (Zhao et al., 2011). In this study, PBPK modeling explained the physiological properties of the four rodent strains by reproducing the observed PK values. In SD rats, the model using software default values deviated substantially from the measured values, and fitting required a delay in T_{max} and a decrease in clearance (Fig. 5A). One potential explanation for this discrepancy is using PEG400 as a solvent, which has been shown to slow absorption in the gastrointestinal tract (Beig et al., 2012), potentially resulting in delayed T_{max}. Corn oil was not applied as a solvent to avoid accidental ingestion during oral administration under anesthesia in wild black rats. Additionally, the predicted metabolic capacity of diphacinone by the ADMET Predictor was much higher than the measured value of hepatic microsomes, necessitating correction for accurate fitting. In S-rats, the clearance was increased, and the gastric transit time was shortened from 5 to 4.5 h (Fig. 5B). The shorter gastric transit time may be due to the difference in organ size between the SD (*Rattus norvegicus*) and black rats (*Rattus rattus*), and the higher C_{max} in the S-rats may be due to a faster absorption rate caused by a shorter gastrointestinal tract. Compared to the SD and S-rats, the C_{max} was higher in S-rats, while $T_{1/2}$ and MRT were shorter, indicating their high excretory capacity. The prolongation of blood coagulation time was stronger in SD rats. Since it has been reported that the symptom of anticoagulant rodenticides in dogs did not correlate with their blood concentration (Waddell et al., 2013), it is considered that the effects of diphacinone are not C_{max}-dependent but excretion-dependent. The comparison among three strains of black rats showed that C_{max} was almost the same in the S- and Bonin rats but was much lower in the R-rat. Modeling of R-rat showed increased hepatic metabolic activity and intestinal first-pass effect compared to the S-rat model, suggesting that R-rat has a high diphacinone metabolic capacity (Fig. 5C). R-rat showed significantly higher CYPs expression in both the liver and gastrointestinal tract (Fig. 4), and significantly higher hepatic metabolic capacity in the warfarin liver perfusion test using R-rat (Takeda et al., 2018), which supports this model. In the Bonin rats, reduced clearance values were required for the fitting compared to S-rats (Fig. 5D). The qPCR analysis also showed significantly lower hepatic *Cyp2c11* expression compared to S-rats, suggesting that hepatic metabolic capacity may be lower (Fig. 4). In addition, fitting to the slightly delayed T_{max} in Bonin rats required extended gastric transit time of 1 h compared to S- and R-rat. This was attributed to the fact that the S−/R- rats had consumed powdered food in captivity, whereas the Bonin rats should have consumed a high fiber plant-based diet, which may have left fiber in the gastrointestinal tract, resulting in delayed absorption. Thus, the PK kinetics of Bonin rats is characterized by slow absorption and slightly

lower excretion than that of S-rats. On the other hand, in the late elimination phase, there was a deviation from the measured values in Bonin rats and R-rats. The PBPK model allows only a single elimination rate coefficient, whereas actual elimination is suggested to be biphasic, with the elimination rate coefficients changing between the early and late elimination phases. In fact, some of second-generation anticoagulant rodenticides are known to persist for a long period of time with a disappearing gradient in the late elimination phase after a single dose (Abi Khalil et al., 2021; Damin-Pernik et al., 2016). Further development of software for PBPK analysis of chemicals with such special pharmacokinetic properties is desirable. Nevertheless, considering that the PBPK modeling for three black rat strains has been generally accomplished, utilization of PBPK modeling by fitting to observed PK data is useful for explaining physiological properties in non-experimental animals.

Rodenticide resistance is typically attributed to VKOR mutations and increased metabolic capacity. The R-rats exhibited a typical resistance phenotype characterized by VKOR mutation, high excretion capacity, and elevated CYP expression levels. R-rats have previously shown high excretory and hepatic metabolic capacity against warfarin as well (Takeda et al., 2018). However, the Bonin rats did not exhibit high excretion capacity *in vivo*, nor did they possess VKOR mutations or elevated CYP expression levels, despite showing PT-INR comparable to that of R-rats. In addition, there are limited reports on *Aifm2*, recently identified as a vitamin K quinone reductase (Mishima et al., 2022), as a factor in the acquisition of rodenticide resistance. In this study, the hepatic expression of *Aifm2* was also quantified, and the expression tended to decrease in all three black rat strains compared to the unexposed control S-rat (Fig. 4), suggesting that diphacinone treatment may have suppressed the expression of *Aifm2*. In particular, the R-rat showed a very low expression level (0.02-fold) compared to the control unexposed group. The meaning of this finding needs to be elucidated through studies on rodenticide-resistant rodents focusing on *Aifm2*. However, no statistically significant difference was observed for the Bonin rats while showing higher *Aifm2* expression than the S-rats ($p = 0.12$). The cause of the low susceptibility to diphacinone in the Bonin rats is unknown and remains to be elucidated. It is interesting to note that the closed colonized S-rats were originally from Chichijima Island before the rodenticide spraying project had begun, allowing for a comparison of the effects of exposure to rodenticides over several decades. A comprehensive comparison of gene mutations and gene expression changes between S-rats and Bonin rats by next-generation sequencing appears to be an effective method to identify unknown mechanisms of resistance acquisition in Bonin rats.

Whether these Bonin rats are truly resistant to rodenticides must be determined by their survival in rodenticide-feeding test proposed by the World Health Organization (WHO, 1981), which was not possible in this study due to ethical considerations regarding animal experimentation. Another limitation of this study is that sampling sites were restricted to the vicinity of the residential area of Chichijima (Fig. 1C) to avoid accidental recapture of endangered birds. A government-led, island-wide susceptibility assessment including lethal feeding tests would be desirable. In this study, 10 μL of whole blood from a single 50 μL blood collection was used to measure PT, with the remainder separated into plasma to obtain 20 μL of plasma. Of this volume, 5 μL could also be used to measure diphacinone and hydroxydiphacinone concentrations. This micro-sampling technique may prove useful for evaluating drug sensitivity in small animals (Takeda et al., 2022). Additionally, although the QuEChERS method typically involves extraction with an organic solvent followed by solid-phase column extraction, good recoveries were obtained without the column extraction (Table 2), indicating that a simple method without column treatment can be used for trace plasma samples. PKPD analysis by the micro-sampling constructed in this study is considered to be a simple and effective method for understanding rodenticide susceptibility and its biological evidence.

5. Conclusion

This study revealed that non-native black rats (Bonin rats) inhabiting Chichijima Island in the Bonin Archipelago in 2022 show only the same level of diphacinone sensitivity as VKOR mutant black rats (R-rats) without VKOR mutation or enhanced metabolic capacity by PKPD analysis and PBPK modeling. Generally, PBPK modeling using GastroPlus has been applied to humans and laboratory animals whose physiological data are built into the software. However, this study applied the PBPK modeling to infer physiological responses to pesticide in non-model/wild rodents by optimizing physiological parameters to fit observed PK. This can be an effective option for wildlife pharmacology/toxicology studies where detailed molecular biology experiments can be difficult.

CRediT authorship contribution statement

Kazuki Takeda: Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Validation, Visualization. **Keita Shimizu:** Data curation, Investigation, Methodology, Validation, Visualization, Writing – original draft. **Miyu Sato:** Investigation, Methodology, Validation, Writing – original draft. **Masafumi Katayama:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Shouta M.M. Nakayama:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing – review & editing. **Kotaro Tanaka:** Investigation. **Yoshinori Ikenaka:** Funding acquisition, Investigation, Validation, Writing – review & editing. **Takuma Hashimoto:** Resources, Writing – review & editing. **Ryuichi Minato:** Resources, Writing – review & editing. **Yusuke Oyamada:** Resources, Writing – review & editing. **Goro Kimura:** Resources, Writing – review & editing. **Tsutomu Tanikawa:** Resources, Writing – review & editing. **Keisuke Kato:** Resources, Writing – review & editing. **Taichi Kusakabe:** Resources, Writing – review & editing. **Mayumi Ishizuka:** Funding acquisition, Supervision, Writing – review & editing. **Ryo Kamata:** Supervision, Writing – review & editing.

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.pestbp.2023.105767.

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