Sensitivity of turtles to anticoagulant rodenticides: risk assessment for green sea turtles (*Chelonia mydas*) in the Ogasawara Islands and comparison of warfarin sensitivity among turtle species

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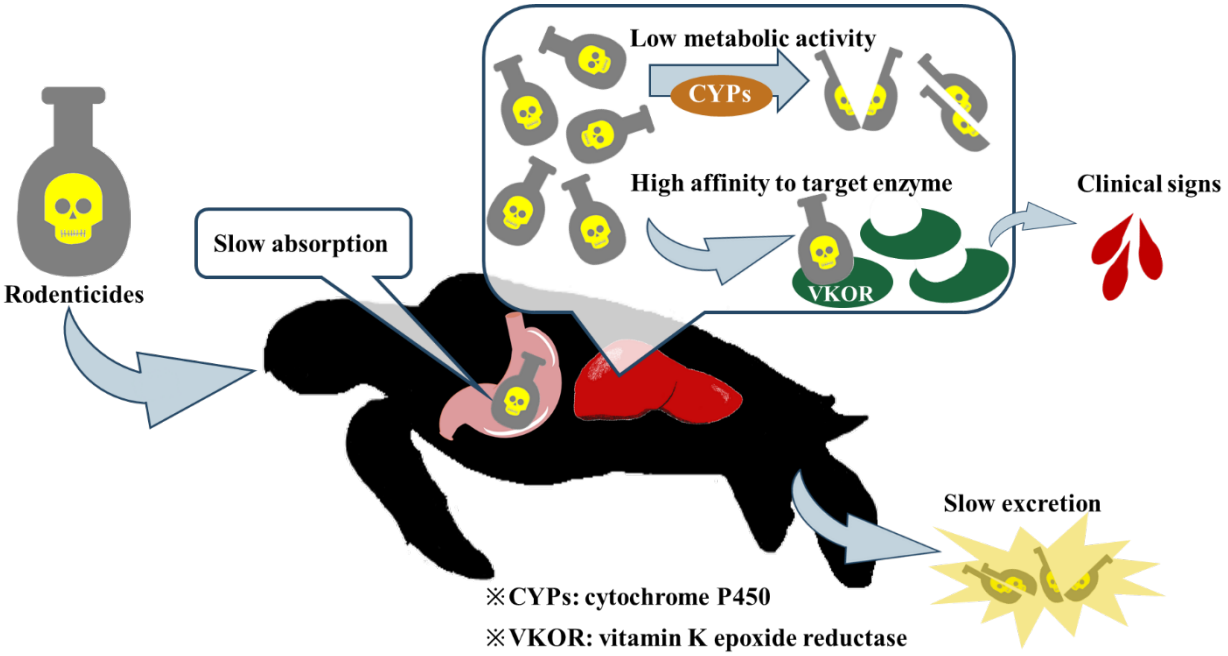
Abstract

Although anticoagulant rodenticides (ARs) are effectively used for the control of invasive rodents, nontarget species are also frequently exposed to ARs and secondary poisonings occur widely. However, little data is available on the effects of ARs, especially on marine organisms. To evaluate the effects of ARs on marine wildlife, we chose green sea turtles (*Chelonia mydas*), which are one of the most common marine organisms around the Ogasawara islands, as our primary study species. The sensitivity of these turtles to ARs was assessed using both *in vivo* and *in vitro* approaches. We administered 4 mg/kg of warfarin sodium either orally or intravenously to juvenile green sea turtles. The turtles exhibited slow pharmacokinetics, and prolongation of prothrombin time (PT) was observed only with intravenous warfarin administration. We also conducted an *in vitro* investigation using liver microsomes from green sea turtles, and two other turtle species (softshell turtle and red-eared slider) and rats. The cytochrome P450 metabolic activity in the liver of green sea turtles was lower than in rats. Additionally, vitamin K epoxide reductase (VKOR), which is the target enzyme of ARs, was inhibited by warfarin in the turtles at lower concentration levels than in rats. These data indicate that turtles may be more sensitive to ARs than rats. We expect that these findings will be helpful for sea turtle conservation following accidental AR-broadcast incidents.

Keywords

Sea turtle; anticoagulant rodenticides; warfarin; vitamin K epoxide reductase; cytochrome P450; risk assessment

Graphical abstract



1. Introduction

In many instances, rodents such as black rats (*Rattus rattus*) and Norway rats (*Rattus norvegicus*) have been artificially introduced to islands, where they have generally caused severe damage to native ecosystems (Towns et al., 2006; Jones et al., 2008). To protect endemic species from invasive rats, rodenticides have often been used as a chemical control method. Anticoagulant rodenticides (ARs) in particular have been used successfully in many countries to reduce rodent populations (Witmer et al., 2007). The target enzyme of ARs is vitamin K 2,3-epoxide reductase (VKOR), which reduces vitamin K 2,3-epoxide (VKO) to vitamin K (Whitlon et al., 1978). Reduced vitamin K is necessary for the activation of blood factors II, VII, IX, and X. ARs inhibit VKOR activity, which leads to a decrease in the level of active vitamin K-dependent blood clotting factors (Kumar et al., 1990). As a result, rats that ingest ARs succumb to chronic bleeding.

However, there are reports that these rodenticides not only cause the intended deaths of rodents, but also kill other wildlife. For example, in the USA, several ARs have been found in the carcasses of raptors such as great horned owls (*Bubo virginianus*) and red-tailed hawks (*Buteo jamaicensis*) (Stone et al., 2000). In New Zealand, 115 lesser short-tailed bats (*Mystacina tuberculata*) were killed by ARs during a rodent control operation (Dennis & Gartrell, 2015). In Spain, ARs were detected in the livers of 38.7% of dead animals that showed signs of hemorrhage (Sánchez-Barbudo et al., 2012). To address the problem of secondary poisoning of nontarget species, many researchers have focused on conducting risk assessments of ARs for wildlife (López and Mateo., 2018).

In general, there are large variations in chemical sensitivity among animal species. For example, the lethal dose of the common AR diphacinose for various bird species differs by 30-fold (Rattner et al., 2012). High sensitivity means a high risk of mortality when that organism is exposed to chemicals. Two parameters are considered important in determining

sensitivity to ARs. The first are the processes of absorption, distribution, metabolism, and excretion (ADME). Initially, ingested ARs are absorbed from the stomach and proximal intestine (Karlyn et al., 2018). They are then transported to the liver and metabolized by various enzymes, including those in the cytochrome P450 (CYP) superfamilies. Finally, the metabolites of ARs are excreted in urine or feces (Breckenridge et al., 1973; Cahill et al., 1979). This series of processes varies widely among animal species. Crowell et al. (2013) noted that the hepatic elimination half-life of diphacinone or coumatetralyl ARs was much longer in cattle than in deer or pigs, and Horak et al. (2018) also mentioned that the half-life of brodifacoum in plasma was much longer in possums than in dogs. The second factor contributing to AR sensitivity is the condition of target enzyme, VKOR. It is well known that AR-resistant human and rats have some amino acid mutations in their VKORs (Rost et al., 2004; Oldenburg et al., 2014). These mutations lead to different 3-dimensional structure of the enzyme and mutant VKORs have unique electron transfer mechanisms (Liu et al., 2014). Some reports mention that amino acid sequence or expression level of VKOR differ depending on the animal species (Nakayama et al., 2020). Thus, these differences may lead to the various sensitivities to ARs among animals.

In addition to ADME and VKOR, it is also helpful to monitor the clinical symptoms caused by ARs. Clinical signs can indicate intoxication without lethality. Measurements of clotting time, especially the prothrombin time (PT) of plasma, have often been used to determine clotting activity in human patients treated with warfarin. This assay is quantitative and is applicable to wildlife, because it is consistent with AR residue levels and the pathogenesis of toxicity (Sage et al., 2010; Rattner et al., 2014).

As elsewhere, there are some areas of Japan in which secondary AR poisonings of wildlife are of concern. The Ogasawara Islands are one area where ARs have been broadly applied for rat eradication. The islands are located in the Pacific Ocean, about 1000 km away from Tokyo, and are home to many endemic species, such as the Bonin flying fox (*Pteropus*

pselaphon) and the red-headed wood pigeon (*Columba janthina nitens*) (Sugita et al, 2009; Ando et al., 2017). In recent years, invasive black rats (*Rattus rattus*) were unintentionally introduced from the mainland via human activity (Shimizu, 2003). These rats have caused severe damage to native species, including seabirds, plants and land snails (Yabe et al., 2009; Chiba et al., 2010). To deal with this problem, the Japanese government has started a rat eradication program using the common AR diphacinone (Hashimoto, 2010).

The Ogasawara Islands constitute one of the largest nesting areas of the green sea turtle (*Chelonia mydas*) in Japan (Kondo et al., 2017). Along the coastlines of the islands, large numbers of these turtles search for nesting beaches. Green sea turtles have a very long life cycle, taking about two decades to reach sexual maturity (Ehrhardt & Witham, 1992). Sea turtles spend most of their life time in the ocean, however, they come up to the land in certain situations such as nesting, basking, and when hatchlings return to the ocean. Thus, there are some possibilities of exposure to various chemical or contaminants for green sea turtles both in the ocean and on land. Moreover, some researchers have already raised concerns that chemicals spilled in the ocean will have adverse effects on sea turtles and lead to population decreases (van de Merwe et al., 2010; Komoroske et al., 2011).

On the Ogasawara Islands, diphacinone has been broadcast in waterproof paper packets. Some of these packets were found in the ocean after the diphacinone had been deployed. Anthropogenic marine debris has been detected in the intestines of stranded sea turtles worldwide (Mascarenhas et al, 2004; Lazar et al., 2011), which indicates that sea turtles sometimes ingest marine debris that they encounter in their natural environment. Therefore, it is also possible that green sea turtles around the Ogasawara Islands may ingest diphacinone packets. However, there have been few risk assessment studies on aquatic organisms, despite reports of AR detection in seawater, living marine fish, and shellfish after the deployment of

ARs on nearby land (Masuda et al., 2015; Pitt et al., 2015; Kotthoff et al., 2018; Regnery et al., 2019).

It is currently unknown whether ingested diphacinone has an adverse effect on turtles. In this study, therefore, we evaluated the green sea turtle's sensitivity to ARs using warfarin. Warfarin was selected for the following reasons. First, warfarin has more background data than diphacinone. It is because warfarin has a long history of use and has a wide range of uses, from rodenticides to human medicines (Lim, 2017). Comparison with previous studies makes it easier to evaluate our data and leads to deeper discussion. Second, warfarin is easier to treat and analyze than diphacinone. Water-solubility of warfarin is higher than that of difacinone and this makes it easier to prepare the dosage solution. Because warfarin and diphacinone have the same mode of action i.e. the inhibition of VKOR followed by the failure of blood coagulation (Lasseur et al., 2007), it is expected that sensitivity to these two compounds is positively correlated. Warfarin is hydroxylated by various CYP superfamilies in the liver (Fig S1) (Daly and King 2003). We used both *in vivo* and *in vitro* methods to evaluate warfarin sensitivity in sea turtles. To obtain information on interspecific differences for ARs, we also used two other species of turtle and Sprague Dawley rats for the *in vitro* investigation. Our findings may be useful in efforts to conserve sea turtle populations in the future.

2. Materials and methods

2.1 Animals

For the *in vivo* exposure experiment, seven living juvenile (yearling) green sea turtles of unknown sex reared in Ogasawara marine center (Tokyo, Japan) were examined in this study (Table 1). Since green sea turtles are rare species all over the world (designated “endangered” by IUCN), we set the sample size as small as possible. Their mean body weight was 2.2 ± 0.14 kg. The turtles were kept in outdoor water tanks (length: 150 cm; width: 130 cm; depth: 60 cm) with water supplied continuously from the sea. Each tank housed two individuals. Water temperatures were monitored using a commercial thermometer (Kenis, Osaka, Japan) during the experiment (Fig. S2). The turtles were fed normal commercial formula food containing mainly fishmeal, krill meal, and shrimp meal. This food was obtained from HIGASHIMARU CO., LTD (Hioki, Japan). The turtles were fasted overnight on the night before warfarin administration.

For the *in vitro* study, we collected fresh livers from each of the animals shown in Table 1. Adult sea turtles used in this experiment were caught in the Ogasawara islands for food by a local fisherman licensed by the Tokyo Metropolitan Water Fisheries Regulation. They were then sacrificed by a local fisherman in a slaughterhouse. Adult male softshell turtles (*Pelodiscus sinensis*) were supplied by a local restaurant in Sapporo (Sapporo, Japan) and sacrificed by a cook in the kitchen. Adult male red-eared slider turtles (*Trachemys scripta elegans*) were obtained from the Municipal Suma Aqualife Park Kobe (Hyogo, Japan). They were euthanized by the injection of pentobarbital. In these three turtle species, all of the collected tissues were immediately placed in liquid nitrogen and kept there while transportation. After arriving at our laboratory, they were stored in a -80 ° C freezer until use. Seven-week-old Sprague Dawley rats (*Rattus norvegicus*) were purchased from Japan SLC (Shizuoka,

Japan) and acclimatized for a week. The rats were housed under a 12/12 h light/dark cycle at 20–23 °C. Food (CE-2; CLEA, Tokyo, Japan) and water were available freely, and they were not fasted before the experiments. After the experiments, the rats were euthanized with an overdose of isoflurane. All these procedures were performed at the Faculty of Veterinary Medicine, Hokkaido University (Sapporo, Japan). All animal care and experimental procedures were performed in accordance with the guidelines of the American Association for Laboratory Animal Care (AAALAC) International (Frederick, Maryland, USA) and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval number: 19-0048).

2.2 Chemicals

The chemicals and reagents obtained from the sources indicated: warfarin metabolites 4′-, 6-, 7-, 8-, and 10-hydroxywarfarin (Ultrafine Chemicals, Manchester, UK); warfarin sodium, ethanol, methanol, diethyl ether, ammonium acetate, acetic acid, sodium citrate, K_2HPO_4, KH_2PO_4, NaOH, and 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (Wako Pure Chemical, Osaka, Japan); and β-glucuronidase, carbamazepine, oxazepam glucuronide, bovine serum albumin (BSA), vitamin K1 epoxide, phenyl-d5-7-hydroxywarfarin, racemic warfarin, pepstatin A, and leupeptin (Sigma–Aldrich, St Louis, MO, USA). We purchased vitamin K1 from Kanto Chemicals (Tokyo, Japan). Vitamin K1-d7 was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Heparin was purchased from Mochida Pharmaceutical (Tokyo, Japan). Sodium pentobarbital was purchased from Kyoritsu Seiyaku (Tokyo, Japan). Tris(hydroxypropyl)phosphine (THP) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.3 Warfarin administration and blood collection

Warfarin administration and blood collection were performed at the Ogasawara Marine Center in July 2019 (Supplementary Figure S3). First, warfarin sodium was dissolved in a saline solution and 4 mg/kg of this solution was administered orally to four of the juvenile green sea turtles using a polyethylene tube (Hibiki polyethylene tubing No. 8) connected to a metal feeding needle (Fuchigami, Kyoto, Japan) and using a 2.5 ml syringe (Terumo, Tokyo, Japan). Brooks et al. (1998) mentioned that oral administration of warfarin (dose: 40 mg/kg) to brown tree snakes (*Boiga irregularis*) produced 80 % mortality. Takeda et al. (2016) reported that oral and intravenous administration of warfarin (dose: 10mg/kg) to rats resulted in prolongation of prothrombin time without death. From these previous studies, we set the administration dose as 4 mg/kg, which is well below the expected LD50 value and at which the effects of warfarin are reliably manifested. We directed the tube through the esophagus and injected the solution directly into the stomach of each turtle. For intravenous administration, the other three juvenile green sea turtles were used. A warfarin solution of 4 mg/kg was administered via the jugular vein using a 2.5 ml syringe and a 25 G needle (Terumo). Blood samples of approximately 600 μl were taken from the jugular vein using a 25 G needle and a 1.0 ml syringe at 5min (0h) and at 1, 2, 4, 6, 12, 24, 48, 72, 96, and 120 h after administration. Each blood sample collected was divided into two tubes. One tube was treated with 3.2% citrate as an anticoagulant for the blood clotting analysis. The other tube was treated with heparin for the measuring of warfarin and metabolite concentrations. Cell-free plasma was prepared by centrifuging whole blood in 1.5 ml microcentrifuge tubes at 2,000 × *g* for 5 min. The plasma samples were temporarily stored at −20 °C at the Ogasawara Marine Center. After the blood collection was complete, the frozen plasma samples were transported to Hokkaido University and stored there at −80 °C until analysis.

Prothrombin time (PT) analysis was performed at Hokkaido University. PT was measured from the 5 min (0 h) and 12, 24, 48, 72, 96, and 120 h blood samples following

Soslau et al. (2004), using PT analysis kits from Diagnostica Stago (Asnières-sur-Seine, France). Briefly, 100 μl of prepared Neoplastine was mixed with 50 μl of the plasma sample in a 1.5 ml microcentrifuge tube for PT analysis. While tapping the tube gently, clot formation was observed visually. The coagulation time was defined as the time at which the first visually observable signs of clot formation appeared. The upper limit was defined as 600 s in this study.

2.4 Warfarin extraction from plasma

Warfarin and hydroxylated warfarin were extracted via liquid–liquid extraction as previously reported (Takeda et al., 2016). Briefly, aliquots of plasma (10 μl) were added to 15 ml centrifuge tubes with 0.1 M sodium acetate (2 ml), 1 μM glucuronidated oxazepam (100 μl, as an internal standard for warfarin and an indicator of deconjugation), 1 μM phenol-d5-7-hydroxywarfarin (10 μl, as an internal standard for hydroxywarfarin), and 4,500 units of β-glucuronidase (100 μl). The mixtures were incubated for 3 h at 37 °C. After incubation, diethyl ether (5 ml) was added to the tubes, which were then vortexed and centrifuged at 3,000 × *g* for 10 min. The organic layer was collected. This procedure was repeated twice. The organic layer was then evaporated to dryness under a gentle stream of N_2 gas. The residue was dissolved in MeOH (200 μl).

2.5 Preparation of liver microsomes

Livers were removed from green sea turtles, softshell turtles, red-eared sliders, and Sprague Dawley rats for the analysis of enzyme activities. The livers were homogenized in 20 ml of homogenization buffer (0.1 M phosphate buffer containing 10% glycerol, 2 mg/l pepstatin A, and 2 mg/l leupeptin). Microsomal fractions were prepared at 4 °C. The supernatant of the first centrifugation at 9,000 × *g* for 20 min was further centrifuged twice at 100,000 × *g* for 60 min. Microsomal pellets were resuspended in resuspension buffer (0.1 M

phosphate buffer containing 10% glycerol, 2 mg/l pepstatin A, and 2 mg/l leupeptin), to provide a protein content of 10 mg/ml, and used to determine CYP activity. The protein concentration of each fraction was measured using the Lowry method (1951) with modifications, and the CYP content was estimated following the method of Omura and Sato (1964).

2.6 Warfarin metabolism

Warfarin metabolism by liver microsomes was analyzed using the method of Fasco et al. (1979) and Takeda et al. (2018) under conditions in which warfarin metabolism was linear. The detail methods are described in SI. Briefly, magnesium chloride (3 mM, final concentration), glucose-6-phosphate (G6P)(5 mM, final concentration), and 10, 25, 50, 100, 200, or 400 μM of warfarin–sodium (final concentration) were mixed and added to a mixture of microsomes (diluted to a final concentration of 1.0 mg protein/ml with potassium phosphate buffer). The total volume of each reaction mixture was 90 μl. Samples were preincubated for 5 min. A 10 μl mixture of glucose-6-phosphate dehydrogenase (G6PDH)(2 IU/ml final concentration) and β-nicotinamide adenine dinucleotide phosphate (β-NADPH) (0.5 mM final concentration) was added to each sample to start the reaction. The reaction was allowed to run for 10 min, then was stopped by adding 1 ml of 100% methanol. In the enzymatic reaction, we set the preincubation and reaction temperature to the physiological conditions for turtles or rats, according to sample type: 37 °C for rats and 25 °C for the three species of turtle. Samples were centrifuged at 15,000 × *g* at 25 °C for 10 min, and the supernatants were transferred into high-performance liquid chromatography (HPLC) vials.

Data on warfarin metabolism were fitted using nonlinear regression to the Michaelis–Menten equation. Estimates of apparent Km and Vmax values were obtained using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

2.7 VKOR activity and inhibition test

The VKOR activity and inhibition assays were performed using the methods of Takeda et al. (2020). Briefly, reaction mixtures were prepared in a HEPES buffer (pH 7.4, 0.1 M), with a total volume of 100 μl. These mixtures contained 1.0 mg/ml liver microsomes and 2, 5, 10, 25, 50, 100, or 300 μM VKO (final concentration). After preincubating samples for 5 min, reactions were started by the addition of THP (1 mM, final concentration). The reactions were continued for 20 min and were finished by the addition of 1 ml of iced diethyl ether. For the inhibition tests, microsomes were diluted in HEPES buffer to a final concentration of 1.0 mg/ml protein. The reaction mixtures (a total volume of 100 μM) contained 50 μM vitamin K1 epoxide and 0, 0.01, 0.05, 0.1, 0.5, 1, or 2.5 μM warfarin sodium (5 μl). The preincubation and reaction temperatures were 37 °C for rats and 25 °C for the three species of turtle.

After stopping the reaction, we added 0.2 μM of vitamin K1-d7 (80 μl) as an internal standard. Vitamin K and VKO were extracted from the reaction mixture using the liquid–liquid extraction method. Liquid–liquid extraction was performed with 5 ml of diethyl ether, and the organic layer was collected and evaporated to dryness under a gentle stream of N_2 gas. The residue was dissolved in 200 μl of methanol.

2.8 HPLC mass spectrometry (MS) conditions

Warfarin and its metabolites were quantified using HPLC coupled with electrospray ionization triple quadrupole mass spectrometry (ESI/MS/MS; LC-8040; Shimadzu, Kyoto, Japan) using a C18 column (Symmetry Shield, RP18 2.1 × 150 mm, 3.5 μm). Vitamin K was analyzed using HPLC coupled with atmosphere pressure chemical ionization triple quadrupole mass spectrometry (APCI/MS/MS, LC 8040; Shimadzu) equipped with a C18 column (Inertsil ODS 3, 2.1 × 150 mm, 5.0 μm). The detail methods described in SI.

2.9 Quality control and quality assurance

Spike and recovery tests with liver samples were performed to investigate recovery rates. The recovery rates for 4′-, 6-, 7-, and 8-OH warfarin were 90.61% ± 25.02% (n = 4), while that of 10-OH warfarin was 57.45% ± 17.00% (n = 4). The recovery rate of warfarin was 108.22% ± 31.72%. The limit of detection (LOD) of OH warfarin was 3.76 nM, and the limit of quantification (LOQ) of OH warfarin was 11.39 nM. For warfarin, the LOD was 87.57 nM and the LOQ was 265.36 nM. For vitamin K quantification, we used the method developed by Takeda et al. (2020). The recovery rates of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 83.89 ± 1.62, 77.89 ± 1.49, and 83.49 ± 1.64 %, respectively (n = 6). The LODs of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 1.40 nM, 5.21 nM, and 3.04 nM, respectively. The LOQs of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 4.24 nM, 15.8 nM, and 9.21 nM, respectively.

2.10 Statistical analysis

The Shapiro–Wilk test showed that the data did not have a normal distribution, and the *F* test showed that the data did not have equal variances. We therefore used nonparametric analyses for all the data. The Steel–Dwass test was used for the comparison of warfarin metabolic activity and VKOR IC_{50} values. The Wilcoxon test was performed to compare the PT values between groups. The Steel test was used to detect changes in the concentration of warfarin and its metabolites in plasma, as well as changes in PT values. In all analyses, $p < 0.05$ was taken to indicate statistical significance. JMP software (version 14; SAS Institute, Cary, NC, USA) was used for the calculations. All values are shown as mean ± standard error (SE).

3. Results

3.1 *In vivo* warfarin metabolism

Plasma warfarin concentrations varied over time after oral (*per os*; p.o.) or intravenous (i.v.) administration (dose: 4mg/kg) (Fig 1). The plasma concentration in the p.o. group was much lower than that of the i.v. group. In the p.o. group, the plasma warfarin concentration had increased by 12 h (0 h: 103.2 ± 125.3 ng/ml; 12 h: 2,340.0 ± 722.7 ng/ml) and it remained at this level throughout the experiment (mean concentration from 24 h to 120 h: 2,085.9 ± 478.9 ng/ml). In contrast, the plasma warfarin concentration in the i.v. group did not vary much (0 h: 14,331.6 ± 1,157.5 ng/ml; 120 h: 10,725.2 ± 226.9 ng/ml) and there were no significant differences between the concentrations at 0 h and the other timepoints (p-values were in the range of 0.40 to 1.00).

In the p.o. group, the plasma concentration of 4′-OH warfarin (one of the metabolites of warfarin) had increased by 96 h (0 h: 16.9 ± 11.9 ng/ml; 96 h: 83.9 ± 31.1 ng/ml) and decreased at 120 h (59.7 ± 11.0 ng/ml). In contrast, the plasma concentration of 4′-OH warfarin in the i.v. group showed a sharp increase by 12 h (0 h: 35.7 ± 12.7 ng/ml; 12 h: 567.6 ± 89.9 ng/ml) and continued to increase until 120 h (120 h: 1,435.0 ± 398.4 ng/ml)(Fig 2). The plasma concentration of 10-OH warfarin, another metabolite of warfarin, generally increased in both groups throughout the experiment although the concentration in the i.v. group was much higher (approximately 10–20 times) than in the p.o. group (Fig 3).

3.2 Coagulation time

PT is an indicator of blood coagulation capacity, so an extended PT indicates prolonged clotting time. .A preliminary test showed that PT of green sea turtles was 144 ± 11 s (n = 8, sex unknown).

With exception of the 5min (0 h) and 12 h time points, the i.v. group showed higher PT values than the p.o. group (Fig 4). This difference may be due to the lower internal dose in the p.o. group compared to the i.v. group. The mean PT values for the p.o. and i.v. groups were 172.0 ± 16.4 s and 241.0 ± 35.5 s, respectively. In particular, the PT of the i.v. group at 120 h (575.7 ± 19.9 s) was significantly higher than that of the p.o. group (263.9 ± 41.6 s; Wilcoxon test $p<0.05$) (Fig 4). Some samples from the i.v. group at 96 and 120 h exceeded the upper limit of 600 s. In contrast, the PT of the p.o. group did not show dramatic changes over the duration of the experiment and there were no significant differences relative to the PT value at 5 min (0 h)(72h: $p = 0.20$, 120h: $p = 0.65$).

3.3 *In vitro* warfarin metabolism

We first checked the effects of temperature on warfarin metabolism in turtles. We used the livers from softshell turtles because the amounts of the liver microsome in this species was enough. In this species, warfarin metabolism was positively related with incubation temperature, and at 30 °C it was approximately 10-fold that at 5 °C (Fig. S5).

We used three turtle species (green sea turtle, Chinese softshell turtle, red-eared slider) and Sprague Dawley rats in our experiment on warfarin metabolism. We assessed metabolic activity based on the CYP content of their microsomes. The CYP content was 195 ± 14.3 pmol/mg (mean ± SE) protein in green sea turtles, 277 ± 23.1 pmol/mg protein in Chinese softshell turtles, 204 ± 43.8 pmol/mg protein in red-eared sliders, and 993 ± 70.8 pmol/mg protein in rats. Of the four species, red-eared sliders showed the highest Vmax/Km values: 8.4 ± 2.3 pmol/min/nmol P450/μM warfarin, followed by rats (5.3 ± 0.38 pmol/min/nmol P450/μM warfarin), and the softshell and green sea turtles showed lower metabolic activity (0.99 ± 0.09 and 1.5 ± 0.15 pmol/min/nmol P450/μM warfarin, respectively; Table 2). However, there were no significant differences among any of these results (rat-green sea turtle: $p = 0.13$, rat-red-

eared slider: $p = 0.16$, rat-softshell turtle: $p = 0.13$, green sea turtle-red-eared slider: $p = 0.53$, green sea turtle-softshell turtle: $p = 0.39$, red-eared slider-softshell turtle: $p = 0.83$). Of the warfarin metabolites, 4′-hydroxylated warfarin was predominant (70–90%) in both turtles and rats (Fig. 5). However, the proportions of the other metabolites clearly differed between the turtles and the rats. Although 10-OH was present in all four species (Fig. 6), the other three metabolites were not (data not shown).In the turtles, 6-OH, 7-OH, and 8-OH warfarin were not detected, except for 6- and 7-OH in the red-eared slider (6-OH, 7-OH: 15.3 ± 5.7 pmol/min/nmol P450). In the rats, however, these metabolites were detected (6-OH, 7-OH: 55.9 ± 15.3 pmol/min/nmol P450; 8-OH: 58.8 ± 11.3 pmol/min/nmol P450).

3.4 *In vitro* VKOR activity assay and inhibition assay

The kinetic parameters of VKOR activity in green sea turtles were measured (Table 3) and plotted in a Michaelis–Menten plot (Fig. S4). In the VKOR inhibition assay, rats and green sea turtles showed similar IC50 values, but there was greater variability among individuals in green sea turtles compared to rats. Although no significant differences were observed, the red-eared sliders and softshell turtles showed more than twice as low values as those of rats (rat-red-eared slider: $p = 0.09$, rat-softshell turtle: $p = 0.13$) (Table 4).

4. Discussion

4.1 Effect of warfarin on green sea turtles

It should be acknowledged that this study used sea water supplied from the coast of the Bonin island, which has not been characterized for the potential presence of other contaminants. Therefore, it cannot be excluded that small amounts of chemicals other than warfarin have been present and may affect the action of warfarin or its metabolism in the body. However, the Bonin island has a low population density (28.4 people /km^2) so there are only few and minor industrial and agricultural activities. Although there is a sewage treatment plant in the bay, it is unlikely to be affected by its wastewater because it is located on the opposite side of the marine center.

The major clinical symptom caused by warfarin is the prolongation of PT. In our study, PT measurements showed that a dose of 4 mg/kg warfarin was not sufficient to cause PT prolongation when administered orally, although significant delays in PT occurred when the dose was administered intravenously (Fig. 4). In response to these results, we can consider several factors. First, it is possible that most of the warfarin administered orally was not absorbed. In the oral administration group, the warfarin and metabolites concentrations varied greatly among individuals, suggesting that some of the warfarin may have been regurgitated underwater. In this experiment, we inserted a polyethylene tube directly into the turtle's esophagus, and this procedure may evoke a regurgitation reflex. Besides technical errors, sex differences in the oral administration group may have contributed to this variability because we did not confirm the sex of individuals in this study. Second, it may take a long time for warfarin to be distributed throughout the body. The PT prolongation may not have been apparent due to the time it takes for warfarin to reach blood circulation.

In contrast to the slow appearance of the effects of the rodenticide in green sea turtles, PT prolongation was detected early in rats. Zhu et al. (1999) and Chu et al. (2011) showed that delayed PT occurred in rats within a day of a single oral warfarin administration (dose: 2 mg/kg and 1 mg/kg, respectively). This time lag in the appearance of the effect of the drug in green sea turtles indicates that warfarin administered orally is absorbed and transported throughout the whole body much more slowly than in rats.

These differences may reflect physiological differences between reptiles and mammals. Amorocho et al. (2008) measured the intake passage time (IPT) in the black sea turtle (*Chelonia mydas agassizii*) using plastic beads, and determined the IPT of the turtles as 23.3 ± 6.6 days. This is much longer than is typical for mammals. For instance, mean digestive marker retention time is 26–27 hours in horses (*Equus ferus caballus*; Orton et al., 1985), 17 hours in rabbits (*Oryctolagus cuniculus*; Sakaguchi et al., 1992), and 8.0 days in manatees (*Trichechus manatus latirostris*; Larkin et al., 2005). Warfarin is usually absorbed from the stomach and proximal intestine (Brophy et al., 2009). Considering the slow IPT in sea turtles, the long absorption time observed in our study makes sense. In addition, the blood respiration rate in reptiles is also slower than in mammals (Sladky & Mans, 2012). The cardiac systems of reptiles differ from those of mammals. Testudines and squamates have two atrial chambers and a single ventricle. They do not have a complete septum in the ventricle, although there is a septum-like structure (Hicks & Wang, 1996). As a result, they normally experience a cardiac shunt, which produces a mixture of oxygenated and deoxygenated blood. Thus, blood circulation efficiency in reptiles is not as high as in mammals, which have a complete interventricular septum (Stephenson et al., 2017). Also, blood pressure in reptiles is generally lower than in mammals. The mean arterial pressure is approximately 4.0 kPa in Chinese softshell turtles (Cho et al., 1988), 5.3 kPa in the South American rattle snake (*Crotalus durissus terrificus*; Bertelsen et al., 2015) and 4.5 kPa in the American alligator (*Alligator*

mississippiensis; Jensen et al., 2016). In contrast, blood pressure is approximately 12 kPa in Wistar rats (*Rattus norvegicus*; Mirhosseini et al., 2016), 8.4 kPa in pigs (*Sus scrofa*; Tuohy et al., 2017), and more than 10 kPa in the horse (Leblanc & Eberhart, 1990). As described above, the slow IPT and unique blood circulation system of reptiles may contribute to slow drug distribution or absorption.

In addition to slow absorption, the amount and longevity of activated blood clotting factors in the body may be another factor. Rattner et al (2014) mentioned that the lag time between exposure and coagulopathy reflects the decreased rates of carboxylation of vitamin K dependent clotting factors and the longevity of carboxylated clotting factors in blood. Although there is little reference on the half-life of clotting factors of reptiles, it is possible that their longevity in the blood is longer than that of mammals.

The life stage of the animals used in this study may also have contributed to the slow pharmacokinetics observed. The turtles used in our *in vivo* study were all juveniles (less than one year old). Generally, ADME and pharmacokinetic drug effects differ between infants or young animals and adults (Milsap & Jusko, 1994). For instance, the concentrations of serum albumin and α1-acid glycoprotein are positively correlated with age (Mazoit & Dalens, 2004). Several other factors, such as a higher ratio of body water (Forman, 1967), also affect the ADME and pharmacokinetics of drugs in young animals. Thus, it is possible that in adult green sea turtles, drug effect will appear earlier than in juveniles but drug toxicity will not last as long as in juveniles. This is because the drug is detoxified and excreted out of the body quickly.

In addition, reptiles such as turtles are not completely homeothermic. The core body temperature of a sea turtle is 0.7–1.7 °C higher than the surrounding seawater temperature (Sato, 2014). The warfarin metabolism in Chinese softshell turtles was affected strongly by incubation temperature, and was positively correlated with temperature we tested (range:5°C to 30°C) (Fig. S5). In our study, the temperature of the water in the tanks fluctuated somewhat during the

experiment, ranging between 26.5 °C and 28.0 °C (Fig. S2). The physical condition of the turtles would have been affected by these changes, and it is possible that lower body temperature slowed blood circulation, suppressed various enzymes activities, and lengthened the time required from warfarin administration to PT change. In our study, the group administered warfarin intravenously showed a significant PT prolongation (Fig. 4). This result indicates that VKOR inhibition may result in a suppression of blood clotting factors in a turtles. In reptiles, the extrinsic blood coagulation pathway appears to play a larger role than the intrinsic pathway (Nevill, 2009). Soslau et al. (2004) demonstrated the presence of blood clotting factors similar to the human factors II, V, VII, and X in sea turtles. In juvenile Chinese softshell turtles dietary vitamin K level was shown to be positively correlated with total plasma prothrombin concentration (Su & Huang, 2019). Taking these previous findings into consideration, we can assume that green sea turtles have vitamin K-dependent blood clotting factors, and that these factors may be activated by VKOR. This suggests that ARs are indeed likely to have similar effects on turtles as they do in rats.

4.2 Warfarin metabolism in green sea turtles

We found that the warfarin concentration of the group dosed orally had increased by 12 h and remained at a constant high level until 120 h (Fig. 1A). In the intravenous group, the warfarin concentration declined slowly, but most of the warfarin nevertheless remained in the blood even at 120 h (Fig. 1B). Thus, in these turtles, the warfarin was not actively metabolized, and it took more than 120 h for it to be excreted.

In contrast, rats given a higher oral dose of warfarin (10 mg/kg) showed a clear decline in warfarin concentration, and most of the warfarin had disappeared from the blood 33 h after administration (Takeda et al., 2016). This supports our conclusion based on the

appearance of prolonged clotting time that the speed of absorption, metabolism, and excretion could be slower in turtles than in rats.

The concentration of warfarin metabolites (4′- and 10-OH warfarin) increased steadily until 120 h in both groups of turtles (Fig. 2 and Fig. 3). This result indicates that the hydroxylation of warfarin does proceed in green sea turtles, albeit slowly. Mallo et al. (2002) performed a pharmacokinetic study by administering the antifungal drug fluconazole to juvenile loggerhead turtles (*Caretta caretta*). They showed that when it was given intravenously, the half-life of fluconazole was 132.6 ± 48.7 h. Lee et al. (1992) administered various doses of fluconazole intravenously to children and showed that its mean half-life was 16.8 ± 1.1 h. This difference indicates that the speed of absorption, metabolism, and excretion is much faster in mammals than in reptiles.

Hulbert and Else (1981) mentioned that the ability to produce energy was three- to six-fold lower in lizards than in rats. Brand et al. (1991) also reported that the standard metabolic rate of rats was seven-fold higher than that of the bearded dragon (*Pogona vitticeps*), and they concluded that this was related to differences in the proton permeability of their mitochondria. In our study, we found that the pharmacokinetics of warfarin in green sea turtles was also slower than in rats, which is consistent with these previous studies. Our *in vitro* study also revealed differences in warfarin metabolism and its metabolite profiles between turtles and rats. In rats, it is well known that various CYP subfamilies are responsible for hydroxylating warfarin. For instance, 4′-OH warfarin is produced by CYP2C11 and CYP2B1, while 10-OH warfarin is produced by CYP3A2 (Fig. S1) (Guengerich et al., 1982). There have been reports on CYP subfamily members in reptiles. For example, CYP 1A- and CYP 2B-like isoforms were detected in several species, such as the American alligator (*Alligator mississippiensis*; Ertl et al., 1998) and the corn snake (*Pantherophis emoryii*; Bani et al., 1998). Another report noted that Kemp’s ridley sea turtles (*Lepidochelys kempii*) had CYP1A, but that its activity level was

low (Gerardo, 2010). Considering that we detected hydroxylated warfarin in green sea turtles, this species might also have some CYP subfamily members, since they play an important role in hydroxylating warfarin. However, the activity or expression levels appear to be relatively low, or their molecular structure may have a much lower binding affinity to warfarin than that of rats. In future experiments, we have to elucidate the CYP status of sea turtles by quantifying the expression levels of CYP isoforms using next-generation RNA sequencing and real-time PCR.

Drug metabolism is also affected by psychophysiological stress. Stress causes some biological responses such as the rise of blood pressure, heart rate, and plasma corticosterone levels (Walker et al., 2012). Since glucocorticoids are involved in the regulation of P450s (Dvorak et al., 2010), the rise of them indirectly changes the drug metabolism. Although it is unclear how much stress was induced by gavage in green sea turtles in our study, it may have affected warfarin metabolism.

We observed interspecific differences in warfarin metabolism among the three turtle species we studied. Vmax/Km values were higher in the red-eared sliders than in the Chinese softshell turtles or green sea turtles (Table 2). This result indicates that red-eared sliders have a greater detoxification capacity when warfarin is present in concentrations that are physiologically tolerated. In general, metabolic activity is correlated with the organisms' feeding habits, and herbivores tend to have a greater detoxification capacity than carnivores, because plants contain various xenobiotics, such as alkaloids or terpenes, that must be metabolized and excreted (McLean et al., 2006). For example, NR1I3 (nuclear receptor subfamily 1 group I member 3), a gene involved in the activation of P450 and UGT1A6, has been confirmed to be deficient in some animals such as killer whale (*Orcinus orca*) (carnivore) and big brown bat (*Eptesicus fuscus*) (insectivore) although this gene exists in naked mole rat (*Heterocephalus glaber*) (herbivore) and cow (*Bos Taurus*) (herbivore) (Hecker et al., 2019).

In the wild, Chinese softshell turtles are mainly carnivorous and feed primarily on insect larvae and small fish (Nuangsaeng & Boonyaratapalin, 2001). In contrast, red-eared sliders are omnivorous and eat a large variety of foods, including animals and plant seeds (Dreslik, 1999; Kimmons & Moll, 2010). Therefore, it is possible that Chinese softshell turtles exhibit lower metabolic activity than red-eared sliders. The red-eared sliders used in our study were originally captured from natural habitats such as rivers and ponds. In the natural environment, turtles might be exposed to a range of chemicals, and some of these might cause CYP induction. Compared to marine animals, the inhabitants of freshwater habitats have a higher risk of exposure to high concentrations of chemicals, because of the lower rate of water flow and smaller total volume of water.

Although green sea turtles are generally herbivorous, they exhibited a low level of warfarin metabolism, similar to Chinese softshell turtles. Richardson et al. (2009) calculated the glutathione S-transferase (GST) activity in four species of sea turtle. They used 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate and found that GST activity was two- to seven-fold lower in sea turtles than in freshwater turtles, such as red-eared sliders. The authors suggested that this difference may be due to differences in osmoregulation capacity, thermoregulation strategy, age at maturation, and home range size. It is possible that some of these differences between freshwater and sea turtles may also contribute to the differences in CYP-mediated warfarin metabolism.

4.3 VKOR activity and inhibition by warfarin

Watanabe et al. (2010) determined the levels of VKOR activity in rats and several species of bird. They found Vmax values that were 14- to 100-fold higher (71.70, 157.6, and 514.5 pmol/min/mg protein for chicken, ostrich, and rat, respectively) than those of the green sea turtles in our study. Additionally, their Km values were more than 30-fold greater (165.8,

187.5, and 176.1 μM for chicken, ostrich, and rat, respectively) than those of green sea turtles. Because of their remarkably low Km, the Vmax/Km values for green sea turtles were higher (1.2 pmol/min/nmol P450/μM warfarin) than for chickens (0.47 pmol/min/nmol P450/μM warfarin) and ostriches (0.87 pmol/min/nmol P450/μM warfarin) but lower than for rats (2.9 pmol/min/nmol P450/μM warfarin). The low Vmax value indicates that green sea turtles may have low VKOR levels. The Vmax/Km value is an indicator of enzyme activity levels at substrate concentrations that are physiologically tolerated. Therefore, VKOR activity levels in green sea turtles seem to be greater than those of birds but lower than those of rats. Generally, green sea turtles feed mainly on algae and seaweed (Carrión-Cortez et al., 2010; Santos et al., 2011), which are rich in vitamin K (Shearer & Newman, 2008). Thus, it is possible that green sea turtles normally ingest sufficient quantities of vitamin K from their food. If they maintain high dietary vitamin K levels in their bodies, they do not need to recycle vitamin K from VKO. This may explain their low levels of VKOR.

The VKOR inhibition test showed that in all three turtle species, warfarin IC_{50} values were lower than in rats, although there were no significant differences between any of the species (Table 4). This could be caused, at least partially, by turtle VKOR having a different molecular structure to that in rats. A low IC_{50} value means that VKOR is easily inhibited by warfarin. Species with low IC_{50} values may thus experience severe adverse effects from the drug. Mauldin et al. (2020) mentioned that turtles and boas exhibited relative insensitivity to ARs such as diphacinone and brodifacoum while lizards such as iguanas seemed to be more sensitive to these chemicals. Even if VKOR is inhibited easily, intoxication will not appear till the activated vitamin K dependent blood clotting factors are used up in the body. Besides the longevity of clotting factors, there may be several complex physiological factors involved in the sensitivity to ARs. There were also differences between the turtle species we studied: the Chinese softshell turtles and red-eared sliders had lower IC_{50} values than the green sea turtles.

To understand VKOR status in turtles, we need to gather more information, such as VKOR sequence data and its expression levels in the body.

5. Conclusions

This study reveals the important aspect of AR sensitivity in green sea turtles. Low liver metabolic activity and the high VKOR affinity to ARs suggest that green sea turtles may suffer from severe adverse effects when they are exposed to ARs. On the other hand, it is unclear how the slow absorption and distribution of ARs affect the actual toxicity to them. Further information is needed to conclusively understand the sensitivity of turtles to ARs, and additional pharmacokinetic parameters, such as half-life, bioavailability, or clearance ability as well as vitamin K source from the food need to be characterized. In addition, molecular biological data such as CYP expression status and the turtles' VKOR amino acid sequence are necessary. For a comprehensive risk assessment, it is also necessary to understand the exposure levels of green sea turtles to diphacinone and their probability of accidental packet ingestion in the natural environment.

6. Ethics statement

All animal care and experimental procedures were performed in accordance with the Guidelines of the AAALAC and approved by the Animal Care and Use Committee of Hokkaido University (approval number: 19-0048).

7. Declaration of Competing Interests

The authors declare that they have no conflicts of interest relating to the work presented in this manuscript.

8. Acknowledgements

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan, via awards made to M.

Ishizuka (No. 16H01779, 18K19847), Y. Ikenaka (18H04132), S.M.M. Nakayama (No. 17KK0009, 20K20633), as well as to the Environment Research and Technology Development Fund (JPMEERF20184R02) of the Environmental Restoration and Conservation Agency of Japan. We also acknowledge financial support from The Soroptimist Japan Foundation, The Nakajima Foundation, The Sumitomo Foundation, The Nihon Seimei Foundation, The Japan Prize Foundation, Hokkaido University SOUSEI Support Program for Young Researchers in FY2020 (SMMN) and Program for supporting introduction of the new sharing system (JPMXS0420100619). This work was technically supported by Mr. Takahiro Ichise and Ms. Nagisa Hirano. We would like to thank Uni-edit (https://uni-edit.net/) for editing and proofreading this manuscript.

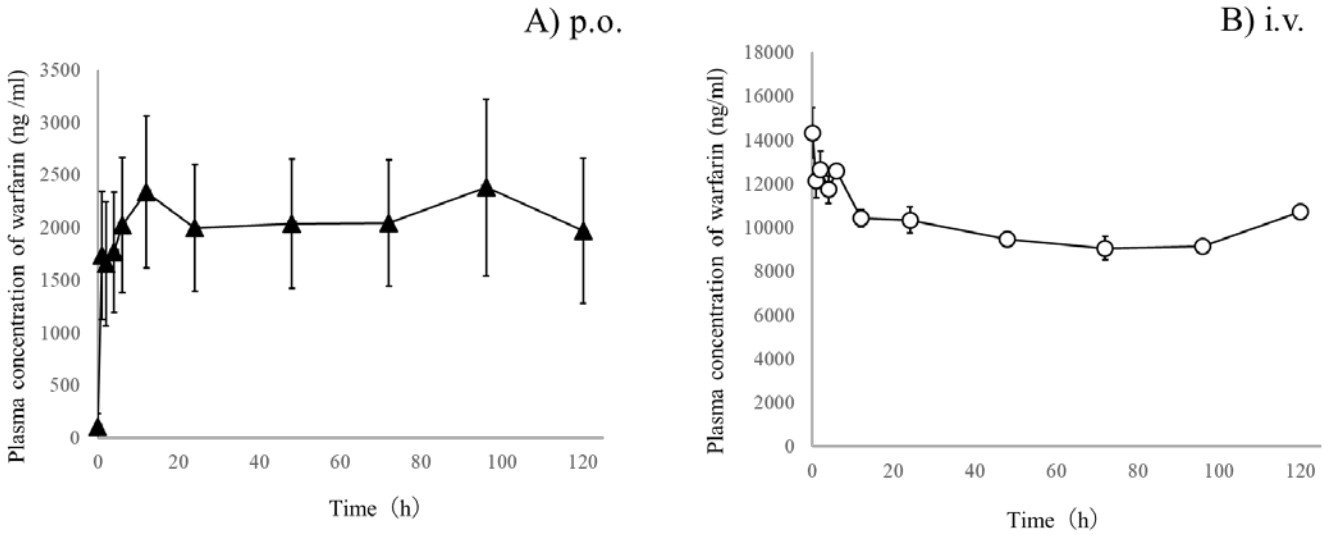


Fig. 1. Time course of changes in plasma warfarin concentration after oral (A) or intravascular (B) administration of 4 mg/kg warfarin. Blood collection was performed at 5 min(0h) and 1, 2, 4, 6, 12, 24, 48, 72, 96, and 120 h after oral administration (p.o.; n = 4) or intravenous administration (i.v.; n = 3). Data are presented as mean (points) ± standard error (error bars).

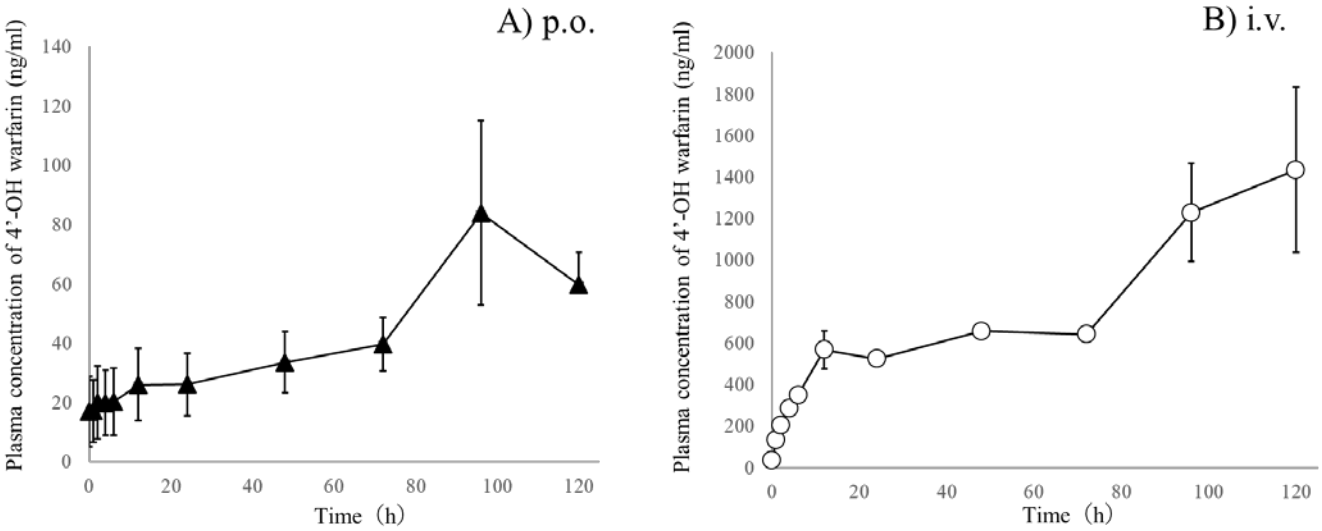


Fig. 2. Time course of changes in plasma 4′-OH warfarin concentration after oral (A) or intravascular (B) administration of 4 mg/kg warfarin. Blood collection was performed at 5 min (0h) and 1, 2, 4, 6, 12, 24, 48, 72, 96, and 120 h after oral administration (p.o.; n = 4) or intravenous administration (i.v.; n = 3). Data are presented as mean (points) ± standard error (error bars).

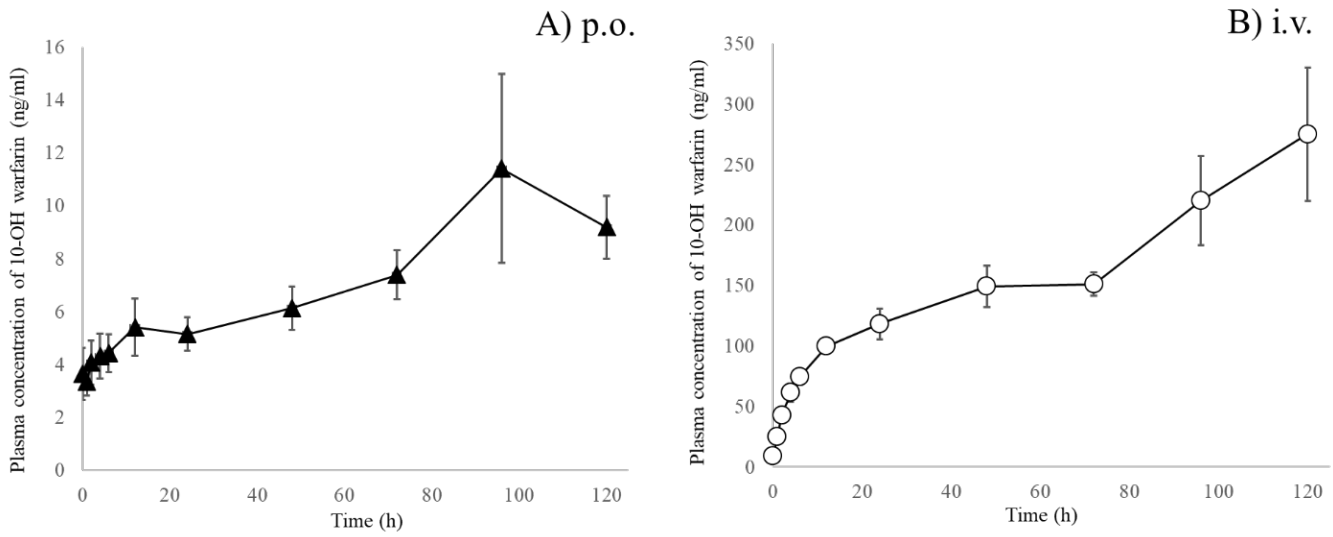


Fig. 3. Time course of changes in plasma 10-OH warfarin concentration after oral (A) or intravascular (B) administration of 4 mg/kg warfarin. Blood collection was performed at 5 min(0h) and 1, 2, 4, 6, 12, 24, 48, 72, 96, and 120 h after oral administration (p.o.; n = 4) or intravenous administration (i.v.; n = 3). Data are presented as mean (points) ± standard error (error bars).

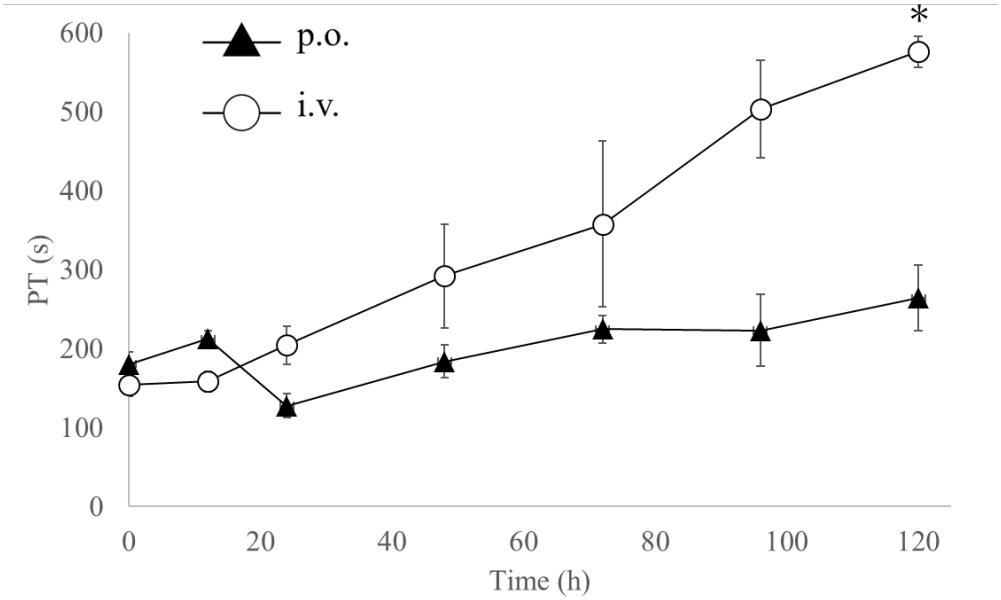


Fig. 4. Prothrombin time (PT) of plasma after oral or intravascular administration of 4 mg/kg warfarin. PT measurement was performed at 5 min (0 h) and 12, 24, 48, 72, 96, and 120 h after administration. The normal PT of green sea turtles is approximately 140 s. We defined the maximum limit of detection as 600 s. Solid triangles represent the values for turtles in the oral administration (p.o.) group (n = 4), and open circles represent those for turtles in the intravenous administration (i.v.) group (n = 3). Data are presented as mean (points) ± standard error (error bars).* $p < 0.05$ (Wilcoxon test, between-group comparisons). Neither group exhibited any significant differences from the value for 0 h ($p > 0.05$; Steel test). However, the value for some of the samples for the i.v. group exceeded the limit of detection (600 s), so it is possible that there were significant differences that we were unable to confirm.

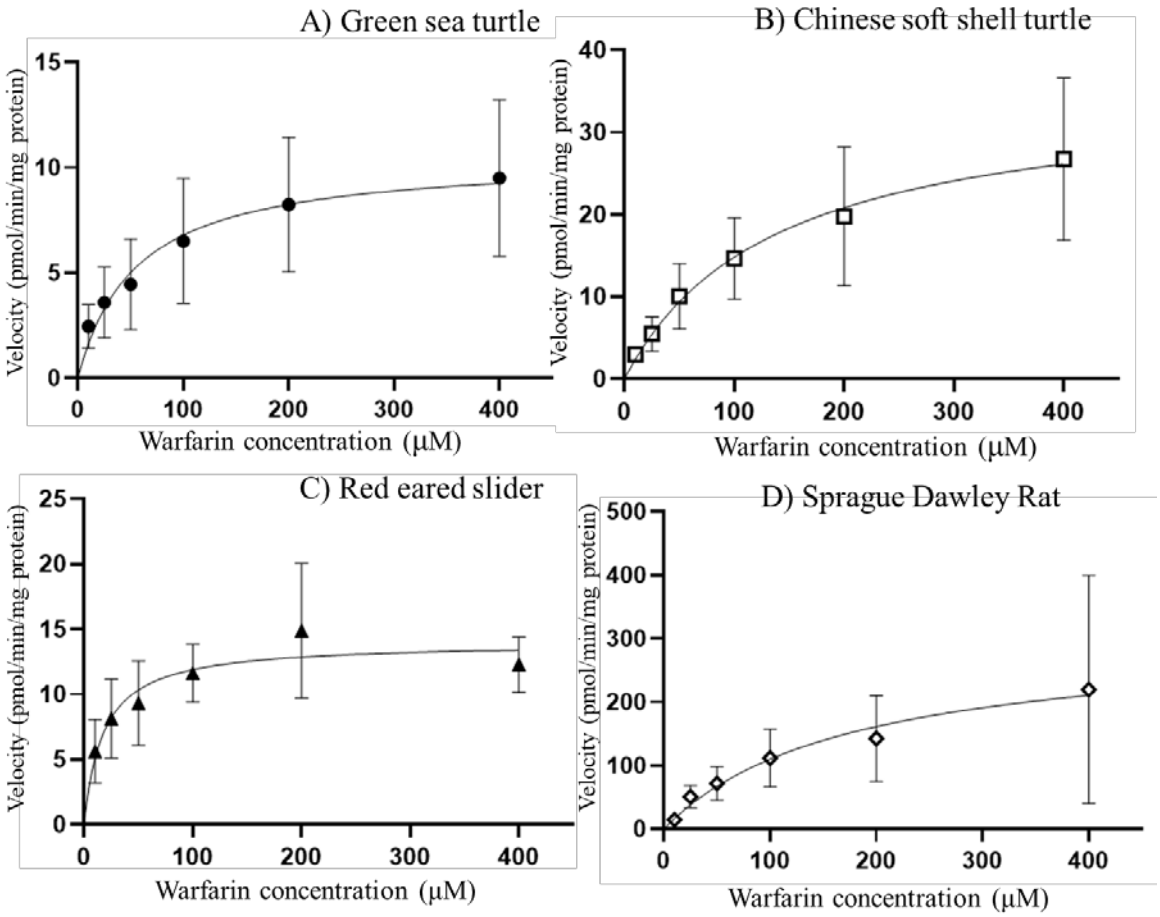


Fig. 5. Michaelis–Menten plots of warfarin 4′-hydroxylation in three turtle species (green sea turtle, Chinese softshell turtle, and red-eared slider) and Sprague Dawley rats. Data are presented as mean (points) ± standard error (error bars).

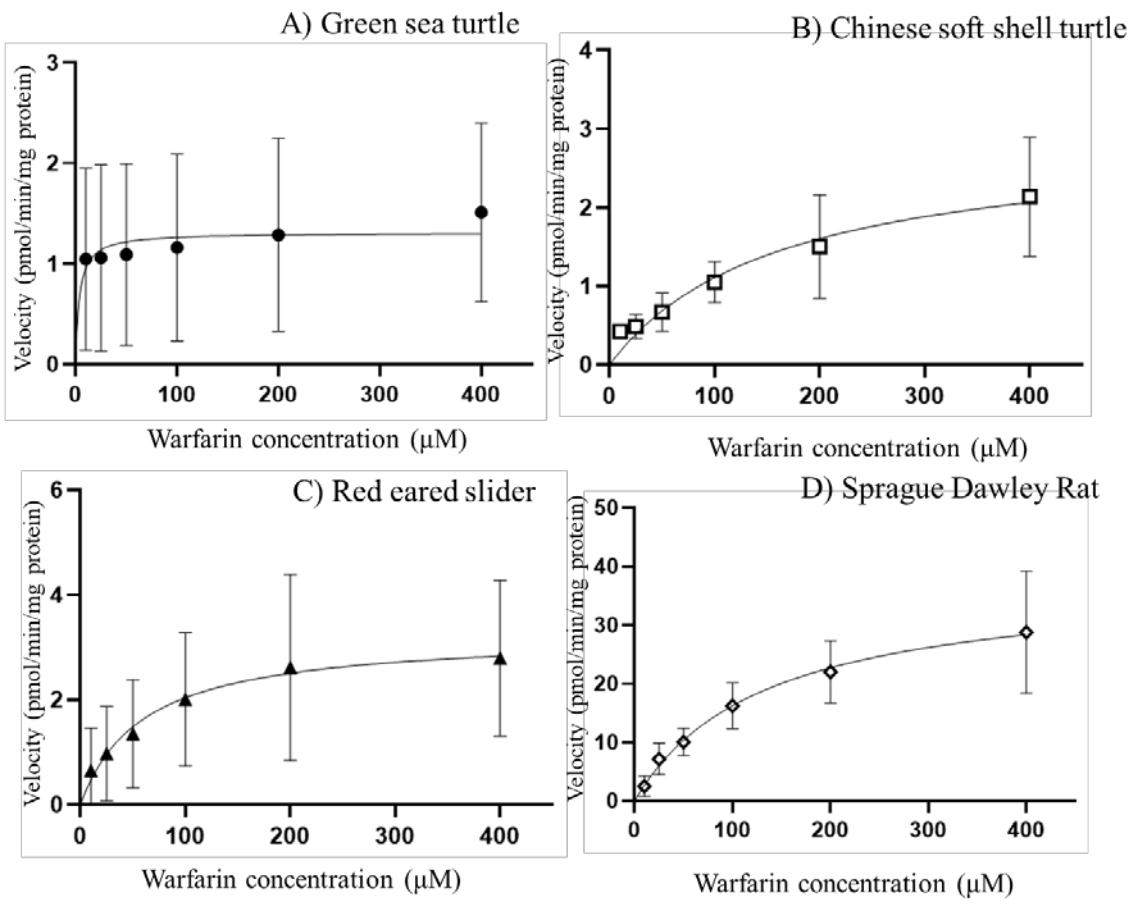


Fig. 6. Michaelis–Menten plots of warfarin 10-hydroxylation in three turtle species (green sea turtle, Chinese softshell turtle, and red-eared slider) and Sprague Dawley rats. Data are presented as mean (points) ± standard error (error bars).

Table 1

Information on animals used in the *in vivo* and *in vitro* experiments

Common name	Scientific name	Sex	Age	Body weight (kg)	Sample size	Source	Use
Green sea turtle	*Chelonia mydas*	unknown	< 1 year	2.2 ± 0.14	7	a	*In vivo* exposure
Green sea turtle	*Chelonia mydas*	male	adult	98.8 ± 3.7	5	b	*In vitro* metabolism & VKOR inhibition test
Chinese softshell turtle	*Pelodiscus sinensis*	male	adult	0.93 ± 0.02	4	c	*In vitro* metabolism & VKOR inhibition test
Red-eared slider	*Trachemys scripta elegans*	male	adult	0.52 ± 0.06	5	d	*In vitro* metabolism & VKOR inhibition test
Sprague Dawley rat	*Rattus norvegicus*	male	7 weeks	205 ± 5 *	5	e	*In vitro* metabolism & VKOR inhibition test

Body weights are presented as mean ± standard error.

* Body weights of rats are expressed in grams (g)

a: Ogasawara Marine Center (Tokyo, Japan)

b: Harvested by local fishermen in Ogasawara Islands (Tokyo, Japan)

c: Local restaurant (Sapporo, Japan)

d: Kobe Municipal Suma Aqualife Park KOBE (Hyogo, Japan)

e: Japan SLC (Shizuoka, Japan)

Table 2

Metabolism of warfarin into its hydroxylated forms, as revealed by the kinetic parameters of hydroxylated warfarin in our four study species

		4'-OH	6-OH, 7-OH	8-OH	10-OH	Total
Sprague Dawley rat (n=5)	Vmax*	166.1 ± 43.6	55.9 ± 14.3	58.8 ± 11.3	32.6 ± 43.6	250.6 ± 39.1
	Km**	75.4 ± 9.8	55.4 ± 11.4	40.7 ± 7.2	139.8 ± 27.4	
	Vmax/Km	2.2 ± 0.12	1.2 ± 0.23	1.1 ± 0.19	0.30 ± 0.01	
Green sea turtle (n=4)	Vmax	50.5 ± 14.2			5.2 ± 2.6	55.8 ± 16.7
	Km	53.5 ± 14.6	ND	ND	17.1 ± 6.6	
	Vmax/Km	1.2 ± 0.16			1.9 ± 1.5	
Chinese softshell turtle (n=4)	Vmax	124.4 ± 12.4			12.7 ± 1.4	137.1 ± 13.7
	Km	135.8 ± 3.4	ND	ND	167.9 ± 13.3	
	Vmax/Km	0.91 ± 0.08			0.08 ± 0.01	
Red-eared slider (n=5)	Vmax	84.7 ± 14.0	15.3 ± 5.7		23.8 ± 8.5	123.8 ± 25.5
	Km	27 ± 12.9	19.5 ± 5.5	ND	105.6 ± 34.9	
	Vmax/Km	6.7 ± 2.1	1.5 ± 0.64		0.50 ± 0.27	

Km and Vmax were calculated according to Michaelis–Menten plots produced in GraphPad Prism 8. Values shown are mean ± standard error. *Vmax: pmol/min/nmol P450 **Km: μM. There were no significant differences between total Vmax/Km values for these species (p >0.05; Steel–Dwass test)(ND, not detected).

Table 3

VKOR activity in green sea turtles

Vmax (pmol/min/ mg protein)	Km (μM)	Vmax/Km
29.6 ± 3.1	4.7 ± 0.7	6.7 ± 0.7

Kinetic parameters of VKOR activity in green sea turtles. The values presented are means ± standard error (n = 5).

Table 4

Warfarin IC_{50}

	IC_{50} (nM)	*p* value			
		S	G	C	R
Sprague Dawley rat (n=5)	147.1 ± 14.6	-	0.98	0.13	0.09
Green sea turtle (n=4)	146.7 ± 46.3	-	-	0.69	0.46
Chinese softshell turtle (n=4)	63.1 ± 6.0	-	-	-	0.83
Red-eared slider (n=5)	55.8 ± 13.0	-	-	-	-

Mean ± standard error IC_{50} (half-maximal inhibitory concentration) values for warfarin. The IC_{50} represents the warfarin concentration that inhibits 50% of VKOR activity. There were no significant differences between any of these species ($p > 0.05$; Steel–Dwass test). S: Sprague Dawley rat, G: Green sea turtle, C: Chinese softshell turtle, R: Red-eared slider

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Supporting information [Materials and methods section]

Sensitivity of turtles to anticoagulant rodenticides: risk assessment for green sea turtles (*Chelonia mydas*) in the Ogasawara Islands and comparison of warfarin sensitivity among turtle species

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2. Materials and methods

2.1 Animals

For the *in vivo* exposure experiment, seven living juvenile (yearling) green sea turtles of unknown sex reared in Ogasawara marine center (Tokyo, Japan) were examined in this study (Table 1). Since green sea turtles are rare species all over the world (designated "endangered" by IUCN), we set the sample size as small as possible. Their mean body weight was 2.2 ± 0.14 kg. The turtles were kept in outdoor water tanks (length: 150 cm; width: 130 cm; depth: 60 cm) with water supplied continuously from the sea. Each tank housed two individuals. Water temperatures were monitored using a commercial thermometer (Kenis, Osaka, Japan) during the experiment (Fig. S2). The turtles were fed normal commercial formula food containing mainly fishmeal, krill meal, and shrimp meal. This food was obtained from HIGASHIMARU CO., LTD (Hioki, Japan). The ~~,~~ turtles~~but~~ were fasted overnight on the night before warfarin administration.

For the *in vitro* study, we collected fresh livers from each of the animals shown in Table 1. Adult sea turtles used in this experiment were caught in the Ogasawara islands for food by a local fisherman licensed by the Tokyo Metropolitan Water Fisheries Regulation. They were then sacrificed by a local fisherman in a slaughterhouse. Adult male softshell turtles (*Pelodiscus sinensis*) were supplied by a local restaurant in Sapporo (Sapporo, Japan) and sacrificed by a cock in the kitchen. Adult male red-eared slider turtles (*Trachemys scripta elegans*) were obtained from the Municipal Suma Aqualife Park Kobe (Hyogo, Japan). They were euthanized by the injection of pentobarbital. Seven-week-old Sprague Dawley rats (*Rattus norvegicus*) were purchased from Japan SLC (Shizuoka, Japan) and acclimatized for a week. The rats were housed under a 12/12 h light/dark cycle at 20–23 °C. Food (CE-2; CLEA, Tokyo, Japan) and water were available freely, and they were not fasted before the

experiments. After the experiments, the rats were euthanized with an overdose of isoflurane. All these procedures were performed at the Faculty of Veterinary Medicine, Hokkaido University (Sapporo, Japan). All animal care and experimental procedures were performed in accordance with the guidelines of the American Association for Laboratory Animal Care (AAALAC) International (Frederick, Maryland, USA~~America~~) and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (approval number: 19-0048).

2.2 Chemicals

The following chemicals and reagents were obtained from the sources indicated: warfarin metabolites 4′-, 6-, 7-, 8-, and 10-hydroxywarfarin (Ultrafine Chemicals, Manchester, UK); warfarin sodium, ethanol, methanol, diethyl ether, ammonium acetate, acetic acid, sodium citrate, K_2HPO_4, KH_2PO_4, NaOH, and 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic acid (HEPES) buffer (Wako Pure Chemical, Osaka, Japan); and β-glucuronidase, carbamazepine, oxazepam glucuronide, bovine serum albumin (BSA), vitamin K1 epoxide, phenyl-d5-7-hydroxywarfarin, racemic warfarin, pepstatin A, and leupeptin (Sigma–Aldrich, St Louis, MO, USA). We purchased vitamin K1 from Kanto Chemicals (Tokyo, Japan). Vitamin K1-d7 was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA). Heparin was purchased from Mochida Pharmaceutical (Tokyo, Japan). Sodium pentobarbital was purchased from Kyoritsu Seiyaku (Tokyo, Japan). Tris(hydroxypropyl)phosphine (THP) was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

2.3 Warfarin administration and blood collection

Warfarin administration and blood collection were performed at the Ogasawara Marine Center in July 2019 (Supplementary Figure S3). First, warfarin sodium was dissolved in a saline solution and 4 mg/kg of this solution was administered orally to four of the juvenile green sea turtles using a polyethylene tube (Hibiki polyethylene tubing No. 8) connected to a metal feeding needle (Fuchigami, Kyoto, Japan) and using a 2.5 ml syringe (Terumo, Tokyo, Japan). Brooks et al. (1998) mentioned that oral administration of warfarin (dose: 40 mg/kg) to brown tree snakes (*Boiga irregularis*) produced 80 % mortality. Takeda et al. (2016) reported that oral and intravenous administration of warfarin (dose: 10mg/kg) to rats resulted in prolongation of prothrombin time without death. From these previous studies, we set the administration dose as 4 mg/kg, which is well below the expected LD50 value and at which the effects of warfarin are reliably manifested. We directed the tube through the esophagus and injected the solution directly into the stomach of each turtle. For intravenous administration, the other three juvenile green sea turtles were used. A warfarin solution of 4 mg/kg was administered via the jugular vein using a 2.5 ml syringe and a 25 G needle (Terumo). Blood samples of approximately 600 μl were taken from the jugular vein using a 25 G needle and a 1.0 ml syringe at 5min (0h) and at 1, 2, 4, 6, 12, 24, 48, 72, 96, and 120 h after administration. Each blood sample collected was divided into two tubes. One tube was treated with 3.2% citrate as an anticoagulant for the blood clotting analysis. The other tube was treated with heparin for the measuring of warfarin and metabolite concentrations. Cell-free plasma was prepared by centrifuging whole blood in 1.5 ml microcentrifuge tubes at 2,000 × *g* for 5 min. The plasma samples were temporarily stored at −20 °C at the Ogasawara Marine Center. After the blood collection was complete, the frozen plasma samples were transported to Hokkaido University and stored there at −80 °C until analysis.

Prothrombin time (PT) analysis was performed at Hokkaido University. PT was measured from the 5 min (0 h) and 12, 24, 48, 72, 96, and 120 h blood samples following

Soslau et al. (2004), using PT analysis kits from Diagnostica Stago (Asnières-sur-Seine, France). Briefly, 100 μl of prepared Neoplastine was mixed with 50 μl of the plasma sample in a 1.5 ml microcentrifuge tube for PT analysis. While tapping the tube gently, clot formation was observed visually. The coagulation time was defined as the time at which the first visually observable signs of clot formation appeared. The upper limit was defined as 600 s in this study.

2.4 Warfarin extraction from plasma

Warfarin and hydroxylated warfarin were extracted via liquid–liquid extraction as previously reported (Takeda et al., 2016). Briefly, aliquots of plasma (10 μl) were added to 15 ml centrifuge tubes with 0.1 M sodium acetate (2 ml), 1 μM glucuronidated oxazepam (100 μl, as an internal standard for warfarin and an indicator of deconjugation), 1 μM phenol-d5-7-hydroxywarfarin (10 μl, as an internal standard for hydroxywarfarin), and 4,500 units of β-glucuronidase (100 μl). The mixtures were incubated for 3 h at 37 °C. After incubation, diethyl ether (5 ml) was added to the tubes, which were then vortexed and centrifuged at 3,000 × *g* for 10 min. The organic layer was collected. This procedure was repeated twice. The organic layer was then evaporated to dryness under a gentle stream of N_2 gas. The residue was dissolved in MeOH (200 μl).

2.5 Preparation of liver microsomes

Livers were removed~~extracted~~ from green sea turtles, softshell turtles, red-eared sliders, and Sprague Dawley rats for the analysis of enzyme activities. The livers were homogenized in 20 ml of homogenization buffer (0.1 M phosphate buffer containing 10% glycerol, 2 mg/l pepstatin A, and 2 mg/l leupeptin). Microsomal fractions were prepared at 4 °C. The supernatant of the first centrifugation at 9,000 × *g* for 20 min was further

centrifuged twice at 100,000 × *g* for 60 min. Microsomal pellets were resuspended in resuspension buffer (0.1 M phosphate buffer containing 10% glycerol, 2 mg/l pepstatin A, and 2 mg/l leupeptin), to provide a protein content of 10 mg/ml, and used to determine CYP activity. The protein concentration of each fraction was measured using the Lowry method (1951) with modifications, and the CYP content was estimated following the method of Omura and Sato (1964).

2.6 Warfarin metabolism

Warfarin metabolism by liver microsomes was analyzed using the method of Fasco et al. (1979) and Takeda et al. (2018) under conditions in which warfarin metabolisme~~c activity~~ was linear. Magnesium chloride (3 mM, final concentration), g~~G~~lucose-6-phosphate (G6P)(5 mM, final concentration), and 10, 25, 50, 100, 200, or 400 μM of warfarin–sodium (final concentration) were mixed and added to a mixture of microsomes (diluted to a final concentration of 1.0 mg protein/ml with potassium phosphate buffer). The total volume of each reaction mixture was 90 μl. Samples were preincubated for 5 min. A 10 μl mixture of g~~G~~lucose-6-phosphate dehydrogenase (G6PDH)(2 IU/ml final concentration) and β-nicotinamide adenine dinucleotide phosphate (β-NADPH) (0.5 mM final concentration) was added to each sample to start the reaction. The reaction was allowed to run for 10 min, then was stopped by adding 1 ml of 100% methanol. In the enzymatic reaction, we set the preincubation and reaction temperature to the physiological conditions for turtles or rats, according to sample type: 37 °C for rats and 25 °C for the three species of turtle. Turtles are ectoterm~~exothermal~~ animals and their activity level and metabolism is greatly affected by surrounding temperatures (Lutz et al., 1989; Litzgus et al., 2003); they can maintain active physiological conditions at around 25 °C (Cabanac et al., 2000). To check the effects of temperature on warfarin metabolism, the metabolic activity of Chinese softshell turtles was

calculated under incubation temperatures of 5–30 °C, increased in increments of 2.5 °C (substrate: 400 μM warfarin sodium). Samples were centrifuged at 15,000 × *g* at 25 °C for 10 min, and the supernatants were transferred into high-performance liquid chromatography (HPLC) vials.

Data on warfarin metabolism were fitted using nonlinear regression to the Michaelis–Menten equation. Estimates of apparent Km and Vmax values were obtained using GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA).

2.7 VKOR activity and inhibition test

The VKOR activity and inhibition assays were performed using the methods of Takeda et al. (2020). Briefly, reaction mixtures were prepared in a HEPES buffer (pH 7.4, 0.1 M), with a total volume of 100 μl. These mixtures contained 1.0 mg/ml liver microsomes and 2, 5, 10, 25, 50, 100, or 300 μM VKO (final concentration). After preincubating samples for 5 min, reactions were started by the addition of THP (1 mM, final concentration). The reactions were continued for 20 min and were finished by the addition of 1 ml of iced diethyl ether. For the inhibition tests, microsomes were diluted in HEPES buffer to a final concentration of 1.0 mg/ml protein. The reaction mixtures (a total volume of 100 μM) contained 50 μM vitamin K1 epoxide and 0, 0.01, 0.05, 0.1, 0.5, 1, or 2.5 μM warfarin sodium (5 μl). The preincubation and reaction temperatures were 37 °C for rats and 25 °C for the three species of turtle.

After stopping the reaction, we added 0.2 μM of vitamin K1-d7 (80 μl) as an internal standard. Vitamin K and VKO were extracted from the reaction mixture using the liquid–liquid extraction method. Liquid–liquid extraction was performed with 5 ml of diethyl ether, and the organic layer was collected and evaporated to dryness under a gentle stream of N_2 gas. The residue was dissolved in 200 μl of methanol.

2.8 HPLC mass spectrometry (MS) conditions

Samples were analyzed using HPLC coupled with electrospray ionization triple quadrupole mass spectrometry (ESI/MS/MS; LC-8040; Shimadzu, Kyoto, Japan) using a C18 column (Symmetry Shield, RP18 2.1 × 150 mm, 3.5 μm). The mobile phase was 10 mM ammonium acetate in 10% MeOH, pH 5.0 (A), and 100% MeOH (B) for warfarin and its metabolites. An injection volume of 5 μl, a flow rate of 0.25 ml/min, and a column temperature of 50 °C were used throughout. In the HPLC, the solvent gradient was as follows: a 20% mobile phase B from 0–2 min, followed by a 20%–90% mobile phase B from 2–15 min, 90% mobile phase B from 15–17 min, and a return to 20% from 17–20 min. The collision energies (CE) and other MS parameters were optimized and are shown in Supplementary Table S1.

For the vitamin K analysis, HPLC coupled with atmosphere pressure chemical ionization triple quadrupole mass spectrometry (APCI/MS/MS, LC-8040; Shimadzu) equipped with a C18 column (Inertsil ODS-3, 2.1 × 150 mm, 5.0 μm) from GL Science (Tokyo, Japan) was used. The mobile phase was 5% 0.1% acetic acid in 95% MeOH (A) and 100% EtOH (B).

The HPLC process followed the methods of Suhara et al. (2005). The CE and other MS parameters were optimized and are shown in Supplementary Table S2, along with the recovery rate of extraction, the limit of detection, and the limit of quantification calculated using the standard curve.

2.9 Quality control and quality assurance

Spike and recovery tests with liver samples were performed to investigate recovery rates. The recovery rates for 4′-, 6-, 7-, and 8-OH warfarin were 90.61% ± 25.02% (n = 4),

while that of 10-OH warfarin was 57.45% ± 17.00% (n = 4). The recovery rate of warfarin was 108.22% ± 31.72%. The limit of detection (LOD) of OH warfarin was 3.76 nM, and the limit of quantification (LOQ) of OH warfarin was 11.39 nM. For warfarin, the LOD was 87.57 nM and the LOQ was 265.36 nM. For vitamin K quantification, we used the method developed by Takeda et al. (2020). The recovery rates of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 83.89 ± 1.62, 77.89 ± 1.49, and 83.49 ± 1.64 %, respectively (n = 6). The LODs of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 1.40 nM, 5.21 nM, and 3.04 nM, respectively. The LOQs of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 4.24 nM, 15.8 nM, and 9.21 nM, respectively.

2.10 Statistical analysis

The Shapiro–Wilk test showed that the data could not be assumed to have a normal distribution, and the *F* test showed that the data could not be assumed to have equal variances. We therefore used nonparametric analyses for all the data. The Steel–Dwass test was used for the comparison of warfarin metabolic activity and VKOR IC_{50} values. The Wilcoxon test was performed to compare the PT values between groups. The Steel test was used to detect changes in the concentration of warfarin and its metabolites in plasma, as well as changes in PT values. In all analyses, $p < 0.05$ was taken to indicate statistical significance. JMP software (version 14; SAS Institute, Cary, NC, USA) was used for the calculations. All values are shown as mean ± standard error (SE).

Supplementary Figures and Tables

Fig. S1. A) Metabolic pathways of warfarin metabolites in rats. Warfarin is hydroxylated by members of the cytochrome P450 superfamily. Various types of CYP are responsible for hydroxylating warfarin. There are five types of warfarin metabolite: 4′-, 6-, 7-, 8-, and 10-OH warfarin. B) Chemical structure of diphacinone which is applied in the Ogasawara islands

Fig. S2. Temperature of the water in the tanks used to house the green sea turtles during the experiment (7–13 July 2019) on the Ogasawara Islands. The water temperature fluctuated between 26.5 °C and 28.0 °C during the experiment.

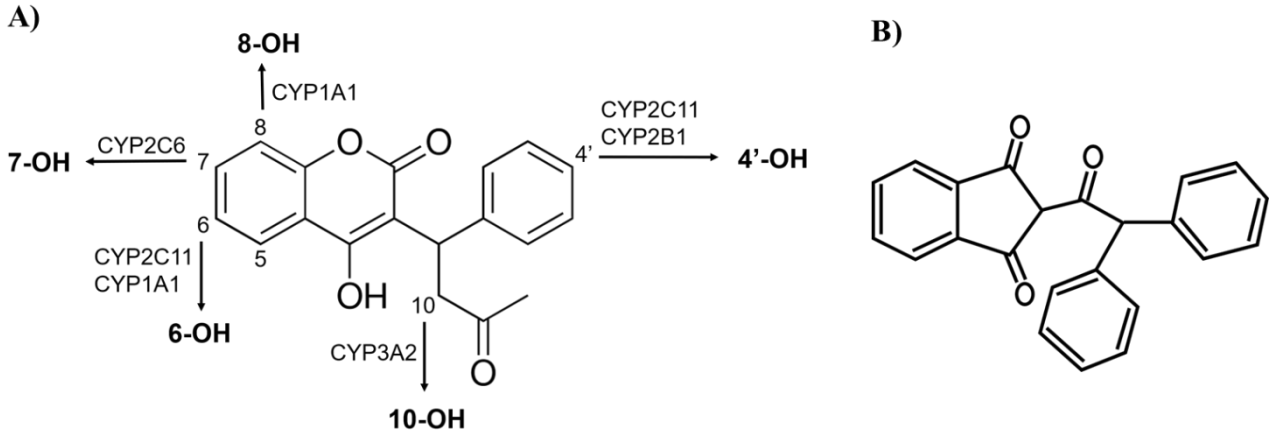
Fig. S3. Photographs of the experiment at the Ogasawara Marine Center (Tokyo, Japan). A: Blood collection from jugular vein. B: Water tank used for the experiment. C: Oral warfarin administration to a juvenile sea turtle using a polyethylene tube.

Fig. S4. Michaelis–Menten plot of VKOR activity in green sea turtles. Data are presented as mean (points) ± standard error (error bars).

Fig. S5. The relationship between incubation temperature and the rate of conversion of warfarin to 4′-OH warfarin in male Chinese softshell turtles (Pelodiscus sinensis) (n = 2). Data are presented as mean (points) ± standard error (error bars). The final substrate (warfarin) concentration was 400 μM. The reaction rate (pmol/min/mg protein) is expressed as a ratio to the rate observed at 25 °C

Table S1. Collision energies and mass spectrometry parameters in the analysis of warfarin and its metabolites

Table S2. Collision energies and mass spectrometry parameters in the Vitamin K analysis



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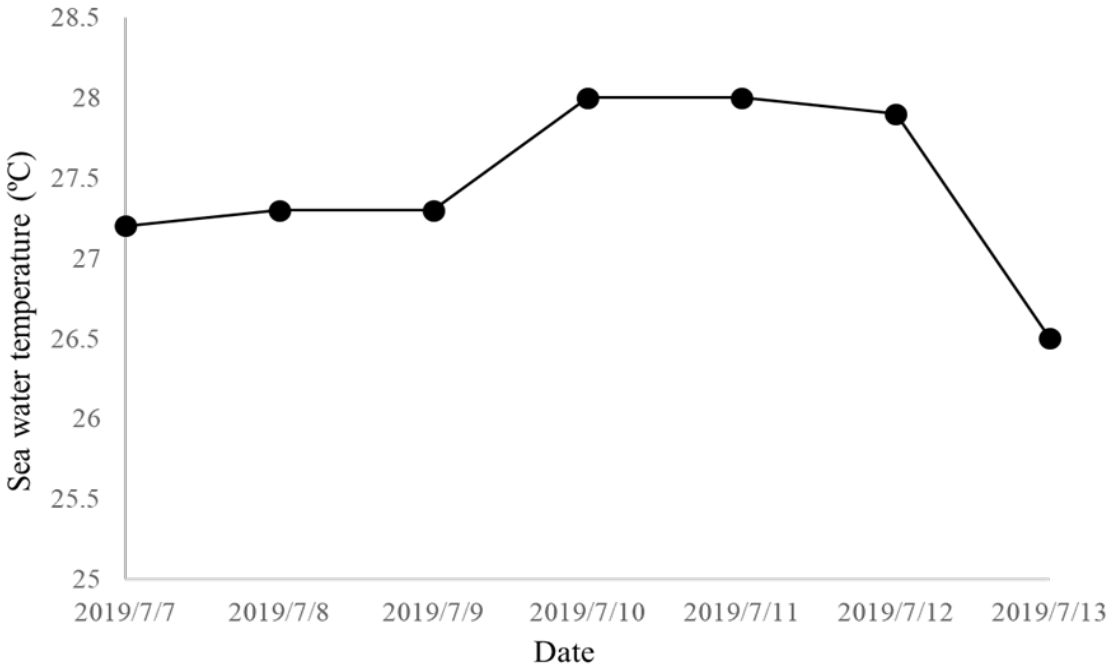

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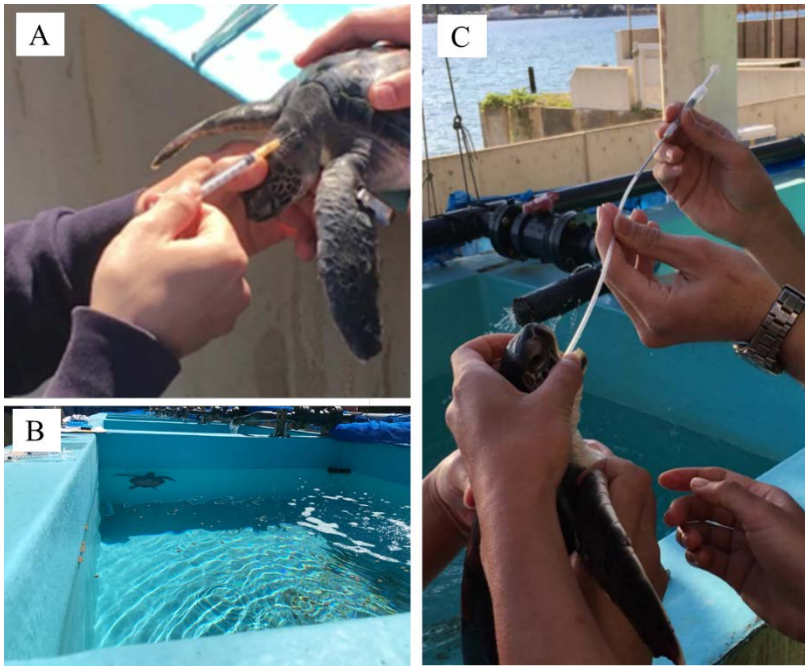

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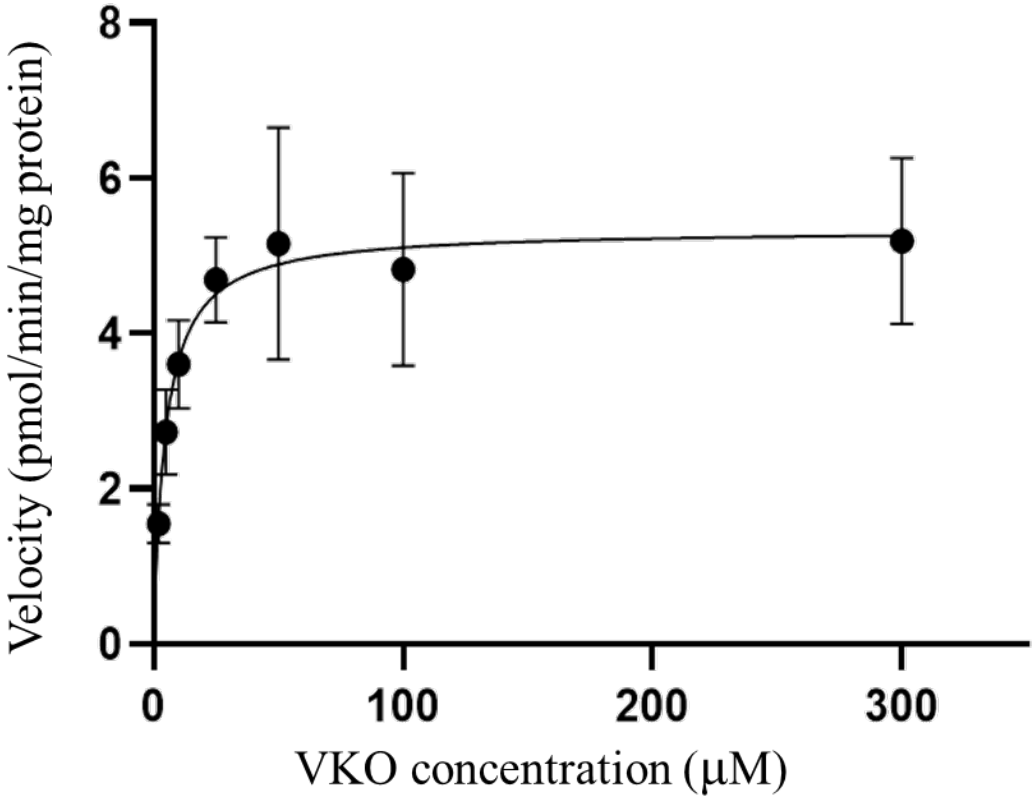

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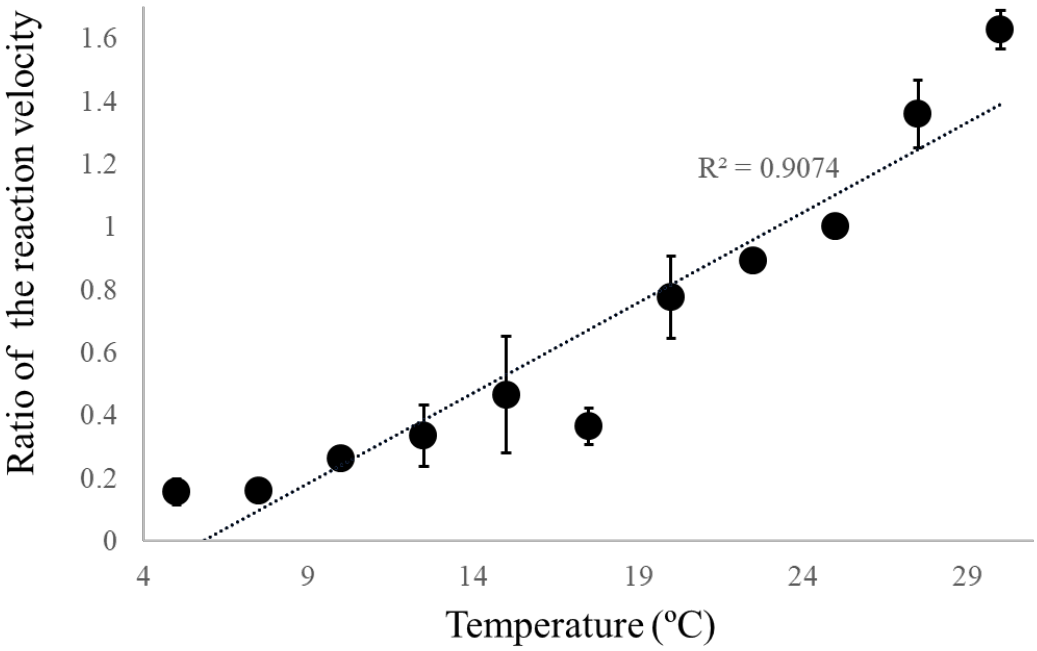

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Table S1. Collision energies and mass spectrometry parameters in the analysis of warfarin and its metabolites

Name		Ionization mode	Precursor product (m/z)	Product (m/z)	Dwell time (ms)	Q1 pre bias (V)	CE	Q3 pre bias (V)
Oxazepam-G (+)	1	+	463	287	100	−23	−15	−28
Oxazepam (+)	2	+	287	241.05	100	−20	−22	−21
Carbamazepine (+)	3	+	237	194	100	−26	−24	−28
7-OH-WF-d5 (−)	4	−	327.9	176.8	100	16	20	17
OH-Warfarin (−)	5	−	323.1	265.2	100	16	24	26
10-OH-Warfarin (−)	6	−	323.1	250.2	100	16	23	25
Warfarin (−)	7	−	307.1	161.25	100	15	21	30

Table S2. Collision energies and mass spectrometry parameters in the Vitamin K analysis

Name		Ionization mode	Precursor product (m/z)	Product (m/z)	Dwell time (ms)	Q1 pre bias (V)	CE	Q3 pre bias (V)
Vitamin K3O	1	−	187.2	159	100	13	22	29
Vitamin K2 Epoxide	2	−	459.1	210.25	100	13	21	21
Vitamin K1 Epoxide	3	−	465.20	421.15	100	13	24	28
Vitamin K2	4	−	443.15	223.05	100	10	34	23
Vitamin K1	5	−	450.20	185.00	100	12	35	18
Vitamin K1-d7	6	−	456.20	438.20	100	12	29	30
Vitamin K3	7	−	172.00	172.10	100	18	30	29