



Title	Sensitivity of turtles to anticoagulant rodenticides: Risk assessment for green sea turtles (<i>Chelonia mydas</i>) in the Ogasawara Islands and comparison of warfarin sensitivity among turtle species
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1 **Sensitivity of turtles to anticoagulant rodenticides: risk assessment for green sea turtles**
2 **(*Chelonia mydas*) in the Ogasawara Islands and comparison of warfarin sensitivity**
3 **among turtle species**

4
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25 **Abstract**

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

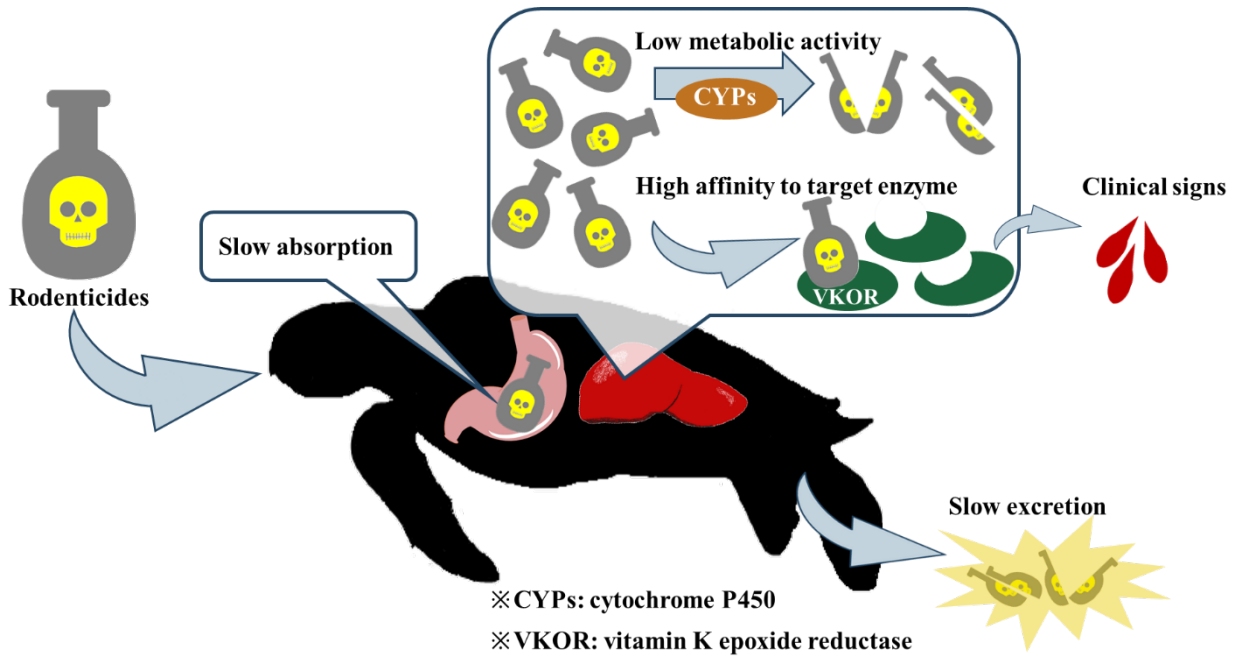
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43 **Keywords**

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

46

47 Graphical abstract



48 **1. Introduction**

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

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

69 In general, there are large variations in chemical sensitivity among animal species.
70 For example, the lethal dose of the common AR diphacinose for various bird species differs by
71 30-fold (Rattner et al., 2012). High sensitivity means a high risk of mortality when that
72 organism is exposed to chemicals. Two parameters are considered important in determining

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

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

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

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

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

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

122 ARs on nearby land (Masuda et al., 2015; Pitt et al., 2015; Kotthoff et al., 2018; Regnery et al.,
123 2019).

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

139 **2. Materials and methods**

140

141 2.1 Animals

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

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

164 Japan) and acclimatized for a week. The rats were housed under a 12/12 h light/dark cycle at
165 20–23 °C. Food (CE-2; CLEA, Tokyo, Japan) and water were available freely, and they were
166 not fasted before the experiments. After the experiments, the rats were euthanized with an
167 overdose of isoflurane. All these procedures were performed at the Faculty of Veterinary
168 Medicine, Hokkaido University (Sapporo, Japan). All animal care and experimental procedures
169 were performed in accordance with the guidelines of the American Association for Laboratory
170 Animal Care (AAALAC) International (Frederick, Maryland, USA) and were approved by the
171 Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido
172 University (approval number: 19-0048).

173

174 2.2 Chemicals

175 The chemicals and reagents obtained from the sources indicated: warfarin
176 metabolites 4'-, 6-, 7-, 8-, and 10-hydroxywarfarin (Ultrafine Chemicals, Manchester, UK);
177 warfarin sodium, ethanol, methanol, diethyl ether, ammonium acetate, acetic acid, sodium
178 citrate, K₂HPO₄, KH₂PO₄, NaOH, and 2-[4-(2-Hydroxyethyl)-1-piperazinyl] ethanesulfonic
179 acid (HEPES) buffer (Wako Pure Chemical, Osaka, Japan); and β-glucuronidase,
180 carbamazepine, oxazepam glucuronide, bovine serum albumin (BSA), vitamin K1 epoxide,
181 phenyl-d5-7-hydroxywarfarin, racemic warfarin, pepstatin A, and leupeptin (Sigma–Aldrich,
182 St Louis, MO, USA). We purchased vitamin K1 from Kanto Chemicals (Tokyo, Japan).
183 Vitamin K1-d7 was obtained from Cambridge Isotope Laboratories (Tewksbury, MA, USA).
184 Heparin was purchased from Mochida Pharmaceutical (Tokyo, Japan). Sodium pentobarbital
185 was purchased from Kyoritsu Seiyaku (Tokyo, Japan). Tris(hydroxypropyl)phosphine (THP)
186 was obtained from Santa Cruz Biotechnology (Dallas, TX, USA).

187

188 2.3 Warfarin administration and blood collection

189 Warfarin administration and blood collection were performed at the Ogasawara
190 Marine Center in July 2019 (Supplementary Figure S3). First, warfarin sodium was dissolved
191 in a saline solution and 4 mg/kg of this solution was administered orally to four of the juvenile
192 green sea turtles using a polyethylene tube (Hibiki polyethylene tubing No. 8) connected to a
193 metal feeding needle (Fuchigami, Kyoto, Japan) and using a 2.5 ml syringe (Terumo, Tokyo,
194 Japan). Brooks et al. (1998) mentioned that oral administration of warfarin (dose: 40 mg/kg)
195 to brown tree snakes (*Boiga irregularis*) produced 80 % mortality. Takeda et al. (2016) reported
196 that oral and intravenous administration of warfarin (dose: 10mg/kg) to rats resulted in
197 prolongation of prothrombin time without death. From these previous studies, we set the
198 administration dose as 4 mg/kg, which is well below the expected LD50 value and at which
199 the effects of warfarin are reliably manifested. We directed the tube through the esophagus and
200 injected the solution directly into the stomach of each turtle. For intravenous administration,
201 the other three juvenile green sea turtles were used. A warfarin solution of 4 mg/kg was
202 administered via the jugular vein using a 2.5 ml syringe and a 25 G needle (Terumo). Blood
203 samples of approximately 600 μ l were taken from the jugular vein using a 25 G needle and a
204 1.0 ml syringe at 5min (0h) and at 1, 2, 4, 6, 12, 24, 48, 72, 96, and 120 h after administration.
205 Each blood sample collected was divided into two tubes. One tube was treated with 3.2% citrate
206 as an anticoagulant for the blood clotting analysis. The other tube was treated with heparin for
207 the measuring of warfarin and metabolite concentrations. Cell-free plasma was prepared by
208 centrifuging whole blood in 1.5 ml microcentrifuge tubes at $2,000 \times g$ for 5 min. The plasma
209 samples were temporarily stored at -20°C at the Ogasawara Marine Center. After the blood
210 collection was complete, the frozen plasma samples were transported to Hokkaido University
211 and stored there at -80°C until analysis.

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

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

219

220 2.4 Warfarin extraction from plasma

221 Warfarin and hydroxylated warfarin were extracted via liquid–liquid extraction as
222 previously reported (Takeda et al., 2016). Briefly, aliquots of plasma (10 μ l) were added to 15
223 ml centrifuge tubes with 0.1 M sodium acetate (2 ml), 1 μ M glucuronidated oxazepam (100 μ l,
224 as an internal standard for warfarin and an indicator of deconjugation), 1 μ M phenol-d5-7-
225 hydroxywarfarin (10 μ l, as an internal standard for hydroxywarfarin), and 4,500 units of β -
226 glucuronidase (100 μ l). The mixtures were incubated for 3 h at 37 °C. After incubation, diethyl
227 ether (5 ml) was added to the tubes, which were then vortexed and centrifuged at 3,000 \times g for
228 10 min. The organic layer was collected. This procedure was repeated twice. The organic layer
229 was then evaporated to dryness under a gentle stream of N₂ gas. The residue was dissolved in
230 MeOH (200 μ l).

231

232 2.5 Preparation of liver microsomes

233 Livers were removed from green sea turtles, softshell turtles, red-eared sliders, and
234 Sprague Dawley rats for the analysis of enzyme activities. The livers were homogenized in 20
235 ml of homogenization buffer (0.1 M phosphate buffer containing 10% glycerol, 2 mg/l
236 pepstatin A, and 2 mg/l leupeptin). Microsomal fractions were prepared at 4 °C. The
237 supernatant of the first centrifugation at 9,000 \times g for 20 min was further centrifuged twice at
238 100,000 \times g for 60 min. Microsomal pellets were resuspended in resuspension buffer (0.1 M

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

243

244 2.6 Warfarin metabolism

245 Warfarin metabolism by liver microsomes was analyzed using the method of Fasco et
246 al. (1979) and Takeda et al. (2018) under conditions in which warfarin metabolism was linear.
247 The detail methods are described in SI. Briefly, magnesium chloride (3 mM, final
248 concentration), glucose-6-phosphate (G6P)(5 mM, final concentration), and 10, 25, 50, 100,
249 200, or 400 μ M of warfarin–sodium (final concentration) were mixed and added to a mixture
250 of microsomes (diluted to a final concentration of 1.0 mg protein/ml with potassium phosphate
251 buffer). The total volume of each reaction mixture was 90 μ l. Samples were preincubated for 5
252 min. A 10 μ l mixture of glucose-6-phosphate dehydrogenase (G6PDH)(2 IU/ml final
253 concentration) and β -nicotinamide adenine dinucleotide phosphate (β -NADPH) (0.5 mM final
254 concentration) was added to each sample to start the reaction. The reaction was allowed to run
255 for 10 min, then was stopped by adding 1 ml of 100% methanol. In the enzymatic reaction, we
256 set the preincubation and reaction temperature to the physiological conditions for turtles or rats,
257 according to sample type: 37 °C for rats and 25 °C for the three species of turtle. Samples were
258 centrifuged at 15,000 \times g at 25 °C for 10 min, and the supernatants were transferred into high-
259 performance liquid chromatography (HPLC) vials.

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

263

264 2.7 VKOR activity and inhibition test

265 The VKOR activity and inhibition assays were performed using the methods of
266 Takeda et al. (2020). Briefly, reaction mixtures were prepared in a HEPES buffer (pH 7.4, 0.1
267 M), with a total volume of 100 μ l. These mixtures contained 1.0 mg/ml liver microsomes and
268 2, 5, 10, 25, 50, 100, or 300 μ M VKO (final concentration). After preincubating samples for 5
269 min, reactions were started by the addition of THP (1 mM, final concentration). The reactions
270 were continued for 20 min and were finished by the addition of 1 ml of iced diethyl ether. For
271 the inhibition tests, microsomes were diluted in HEPES buffer to a final concentration of 1.0
272 mg/ml protein. The reaction mixtures (a total volume of 100 μ M) contained 50 μ M vitamin K1
273 epoxide and 0, 0.01, 0.05, 0.1, 0.5, 1, or 2.5 μ M warfarin sodium (5 μ l). The preincubation and
274 reaction temperatures were 37 °C for rats and 25 °C for the three species of turtle.

275 After stopping the reaction, we added 0.2 μ M of vitamin K1-d7 (80 μ l) as an internal
276 standard. Vitamin K and VKO were extracted from the reaction mixture using the liquid–liquid
277 extraction method. Liquid–liquid extraction was performed with 5 ml of diethyl ether, and the
278 organic layer was collected and evaporated to dryness under a gentle stream of N₂ gas. The
279 residue was dissolved in 200 μ l of methanol.

280

281 2.8 HPLC mass spectrometry (MS) conditions

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

288

289 2.9 Quality control and quality assurance

290 Spike and recovery tests with liver samples were performed to investigate recovery
291 rates. The recovery rates for 4'-, 6-, 7-, and 8-OH warfarin were $90.61\% \pm 25.02\%$ ($n = 4$),
292 while that of 10-OH warfarin was $57.45\% \pm 17.00\%$ ($n = 4$). The recovery rate of warfarin was
293 $108.22\% \pm 31.72\%$. The limit of detection (LOD) of OH warfarin was 3.76 nM, and the limit
294 of quantification (LOQ) of OH warfarin was 11.39 nM. For warfarin, the LOD was 87.57 nM
295 and the LOQ was 265.36 nM. For vitamin K quantification, we used the method developed by
296 Takeda et al. (2020). The recovery rates of vitamin K1, vitamin K1 epoxide, and vitamin K1-
297 d7 were 83.89 ± 1.62 , 77.89 ± 1.49 , and $83.49 \pm 1.64\%$, respectively ($n = 6$). The LODs of
298 vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 1.40 nM, 5.21 nM, and 3.04 nM,
299 respectively. The LOQs of vitamin K1, vitamin K1 epoxide, and vitamin K1-d7 were 4.24 nM,
300 15.8 nM, and 9.21 nM, respectively.

301

302 2.10 Statistical analysis

303 The Shapiro–Wilk test showed that the data did not have a normal distribution, and
304 the F test showed that the data did not have equal variances. We therefore used nonparametric
305 analyses for all the data. The Steel–Dwass test was used for the comparison of warfarin
306 metabolic activity and VKOR IC_{50} values. The Wilcoxon test was performed to compare the
307 PT values between groups. The Steel test was used to detect changes in the concentration of
308 warfarin and its metabolites in plasma, as well as changes in PT values. In all analyses, $p <$
309 0.05 was taken to indicate statistical significance. JMP software (version 14; SAS Institute,
310 Cary, NC, USA) was used for the calculations. All values are shown as mean \pm standard error
311 (SE).

312 3. Results

313

314 3.1 *In vivo* warfarin metabolism

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

324 In the p.o. group, the plasma concentration of 4'-OH warfarin (one of the
325 metabolites of warfarin) had increased by 96 h (0 h: 16.9 ± 11.9 ng/ml; 96 h: 83.9 ± 31.1 ng/ml)
326 and decreased at 120 h (59.7 ± 11.0 ng/ml). In contrast, the plasma concentration of 4'-OH
327 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
328 ± 89.9 ng/ml) and continued to increase until 120 h (120 h: $1,435.0 \pm 398.4$ ng/ml)(Fig 2). The
329 plasma concentration of 10-OH warfarin, another metabolite of warfarin, generally increased
330 in both groups throughout the experiment although the concentration in the i.v. group was much
331 higher (approximately 10–20 times) than in the p.o. group (Fig 3).

332

333 3.2 Coagulation time

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

337 With exception of the 5min (0 h) and 12 h time points, the i.v. group showed higher
338 PT values than the p.o. group (Fig 4). This difference may be due to the lower internal dose in
339 the p.o. group compared to the i.v. group. The mean PT values for the p.o. and i.v. groups were
340 172.0 ± 16.4 s and 241.0 ± 35.5 s, respectively. In particular, the PT of the i.v. group at 120 h
341 (575.7 ± 19.9 s) was significantly higher than that of the p.o. group (263.9 ± 41.6 s; Wilcoxon
342 test $p < 0.05$) (Fig 4). Some samples from the i.v. group at 96 and 120 h exceeded the upper limit
343 of 600 s. In contrast, the PT of the p.o. group did not show dramatic changes over the duration
344 of the experiment and there were no significant differences relative to the PT value at 5 min (0
345 h)(72h: $p = 0.20$, 120h: $p = 0.65$).

346

347 3.3 *In vitro* warfarin metabolism

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

352 We used three turtle species (green sea turtle, Chinese softshell turtle, red-eared
353 slider) and Sprague Dawley rats in our experiment on warfarin metabolism. We assessed
354 metabolic activity based on the CYP content of their microsomes. The CYP content was $195 \pm$
355 14.3 pmol/mg (mean \pm SE) protein in green sea turtles, 277 ± 23.1 pmol/mg protein in Chinese
356 softshell turtles, 204 ± 43.8 pmol/mg protein in red-eared sliders, and 993 ± 70.8 pmol/mg
357 protein in rats. Of the four species, red-eared sliders showed the highest V_{max}/K_m values: 8.4
358 ± 2.3 pmol/min/nmol P450/ μ M warfarin, followed by rats (5.3 ± 0.38 pmol/min/nmol P450/ μ M
359 warfarin), and the softshell and green sea turtles showed lower metabolic activity (0.99 ± 0.09
360 and 1.5 ± 0.15 pmol/min/nmol P450/ μ M warfarin, respectively; Table 2). However, there were
361 no significant differences among any of these results (rat-green sea turtle: $p = 0.13$, rat-red-

362 eared slider: $p = 0.16$, rat-softshell turtle: $p = 0.13$, green sea turtle-red-eared slider: $p = 0.53$,
363 green sea turtle-softshell turtle: $p = 0.39$, red-eared slider-softshell turtle: $p = 0.83$). Of the
364 warfarin metabolites, 4'-hydroxylated warfarin was predominant (70–90%) in both turtles and
365 rats (Fig. 5). However, the proportions of the other metabolites clearly differed between the
366 turtles and the rats. Although 10-OH was present in all four species (Fig. 6), the other three
367 metabolites were not (data not shown). In the turtles, 6-OH, 7-OH, and 8-OH warfarin were not
368 detected, except for 6- and 7-OH in the red-eared slider (6-OH, 7-OH: 15.3 ± 5.7
369 pmol/min/nmol P450). In the rats, however, these metabolites were detected (6-OH, 7-OH:
370 55.9 ± 15.3 pmol/min/nmol P450; 8-OH: 58.8 ± 11.3 pmol/min/nmol P450).

371

372 3.4 *In vitro* VKOR activity assay and inhibition assay

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

379 **4. Discussion**

380

381 4.1 Effect of warfarin on green sea turtles

382 It should be acknowledged that this study used sea water supplied from the coast of
383 the Bonin island, which has not been characterized for the potential presence of other
384 contaminants. Therefore, it cannot be excluded that small amounts of chemicals other than
385 warfarin have been present and may affect the action of warfarin or its metabolism in the body.
386 However, the Bonin island has a low population density (28.4 people /km²) so there are only
387 few and minor industrial and agricultural activities. Although there is a sewage treatment plant
388 in the bay, it is unlikely to be affected by its wastewater because it is located on the opposite
389 side of the marine center.

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

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

409 These differences may reflect physiological differences between reptiles and
410 mammals. Amorocho et al. (2008) measured the intake passage time (IPT) in the black sea
411 turtle (*Chelonia mydas agassizii*) using plastic beads, and determined the IPT of the turtles as
412 23.3 ± 6.6 days. This is much longer than is typical for mammals. For instance, mean digestive
413 marker retention time is 26–27 hours in horses (*Equus ferus caballus*; Orton et al., 1985), 17
414 hours in rabbits (*Oryctolagus cuniculus*; Sakaguchi et al., 1992), and 8.0 days in manatees
415 (*Trichechus manatus latirostris*; Larkin et al., 2005). Warfarin is usually absorbed from the
416 stomach and proximal intestine (Brophy et al., 2009). Considering the slow IPT in sea turtles,
417 the long absorption time observed in our study makes sense. In addition, the blood respiration
418 rate in reptiles is also slower than in mammals (Sladky & Mans, 2012). The cardiac systems of
419 reptiles differ from those of mammals. Testudines and squamates have two atrial chambers and
420 a single ventricle. They do not have a complete septum in the ventricle, although there is a
421 septum-like structure (Hicks & Wang, 1996). As a result, they normally experience a cardiac
422 shunt, which produces a mixture of oxygenated and deoxygenated blood. Thus, blood
423 circulation efficiency in reptiles is not as high as in mammals, which have a complete
424 interventricular septum (Stephenson et al., 2017). Also, blood pressure in reptiles is generally
425 lower than in mammals. The mean arterial pressure is approximately 4.0 kPa in Chinese
426 softshell turtles (Cho et al., 1988), 5.3 kPa in the South American rattle snake (*Crotalus*
427 *durissus terrificus*; Bertelsen et al., 2015) and 4.5 kPa in the American alligator (*Alligator*

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

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

439 The life stage of the animals used in this study may also have contributed to the slow
440 pharmacokinetics observed. The turtles used in our *in vivo* study were all juveniles (less than
441 one year old). Generally, ADME and pharmacokinetic drug effects differ between infants or
442 young animals and adults (Milsap & Jusko, 1994). For instance, the concentrations of serum
443 albumin and α 1-acid glycoprotein are positively correlated with age (Mazoit & Dalens, 2004).
444 Several other factors, such as a higher ratio of body water (Forman, 1967), also affect the
445 ADME and pharmacokinetics of drugs in young animals. Thus, it is possible that in adult green
446 sea turtles, drug effect will appear earlier than in juveniles but drug toxicity will not last as long
447 as in juveniles. This is because the drug is detoxified and excreted out of the body quickly.

448 In addition, reptiles such as turtles are not completely homeothermic. The core body
449 temperature of a sea turtle is 0.7–1.7 °C higher than the surrounding seawater temperature (Sato,
450 2014). The warfarin metabolism in Chinese softshell turtles was affected strongly by incubation
451 temperature, and was positively correlated with temperature we tested (range:5°C to 30°C) (Fig.
452 S5). In our study, the temperature of the water in the tanks fluctuated somewhat during the

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

467

468 4.2 Warfarin metabolism in green sea turtles

469 We found that the warfarin concentration of the group dosed orally had increased by
470 12 h and remained at a constant high level until 120 h (Fig. 1A). In the intravenous group, the
471 warfarin concentration declined slowly, but most of the warfarin nevertheless remained in the
472 blood even at 120 h (Fig. 1B). Thus, in these turtles, the warfarin was not actively metabolized,
473 and it took more than 120 h for it to be excreted.

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

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

479 The concentration of warfarin metabolites (4'- and 10-OH warfarin) increased
480 steadily until 120 h in both groups of turtles (Fig. 2 and Fig. 3). This result indicates that the
481 hydroxylation of warfarin does proceed in green sea turtles, albeit slowly. Mallo et al. (2002)
482 performed a pharmacokinetic study by administering the antifungal drug fluconazole to
483 juvenile loggerhead turtles (*Caretta caretta*). They showed that when it was given
484 intravenously, the half-life of fluconazole was 132.6 ± 48.7 h. Lee et al. (1992) administered
485 various doses of fluconazole intravenously to children and showed that its mean half-life was
486 16.8 ± 1.1 h. This difference indicates that the speed of absorption, metabolism, and excretion
487 is much faster in mammals than in reptiles.

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

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

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

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

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

537 Although green sea turtles are generally herbivorous, they exhibited a low level of
538 warfarin metabolism, similar to Chinese softshell turtles. Richardson et al. (2009) calculated
539 the glutathione S-transferase (GST) activity in four species of sea turtle. They used 1-chloro-
540 2,4-dinitrobenzene (CDNB) as a substrate and found that GST activity was two- to seven-fold
541 lower in sea turtles than in freshwater turtles, such as red-eared sliders. The authors suggested
542 that this difference may be due to differences in osmoregulation capacity, thermoregulation
543 strategy, age at maturation, and home range size. It is possible that some of these differences
544 between freshwater and sea turtles may also contribute to the differences in CYP-mediated
545 warfarin metabolism.

546

547 4.3 VKOR activity and inhibition by warfarin

548 Watanabe et al. (2010) determined the levels of VKOR activity in rats and several
549 species of bird. They found V_{max} values that were 14- to 100-fold higher (71.70, 157.6, and
550 514.5 pmol/min/mg protein for chicken, ostrich, and rat, respectively) than those of the green
551 sea turtles in our study. Additionally, their K_m values were more than 30-fold greater (165.8,

552 187.5, and 176.1 μM for chicken, ostrich, and rat, respectively) than those of green sea turtles.
553 Because of their remarkably low K_m , the V_{max}/K_m values for green sea turtles were higher
554 (1.2 pmol/min/nmol P450/ μM warfarin) than for chickens (0.47 pmol/min/nmol P450/ μM
555 warfarin) and ostriches (0.87 pmol/min/nmol P450/ μM warfarin) but lower than for rats (2.9
556 pmol/min/nmol P450/ μM warfarin). The low V_{max} value indicates that green sea turtles may
557 have low VKOR levels. The V_{max}/K_m value is an indicator of enzyme activity levels at
558 substrate concentrations that are physiologically tolerated. Therefore, VKOR activity levels in
559 green sea turtles seem to be greater than those of birds but lower than those of rats. Generally,
560 green sea turtles feed mainly on algae and seaweed (Carrión-Cortez et al., 2010; Santos et al.,
561 2011), which are rich in vitamin K (Shearer & Newman, 2008). Thus, it is possible that green
562 sea turtles normally ingest sufficient quantities of vitamin K from their food. If they maintain
563 high dietary vitamin K levels in their bodies, they do not need to recycle vitamin K from VKO.
564 This may explain their low levels of VKOR.

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

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

579 **5. Conclusions**

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

591

592 **6. Ethics statement**

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

596

597 **7. Declaration of Competing Interests**

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

600

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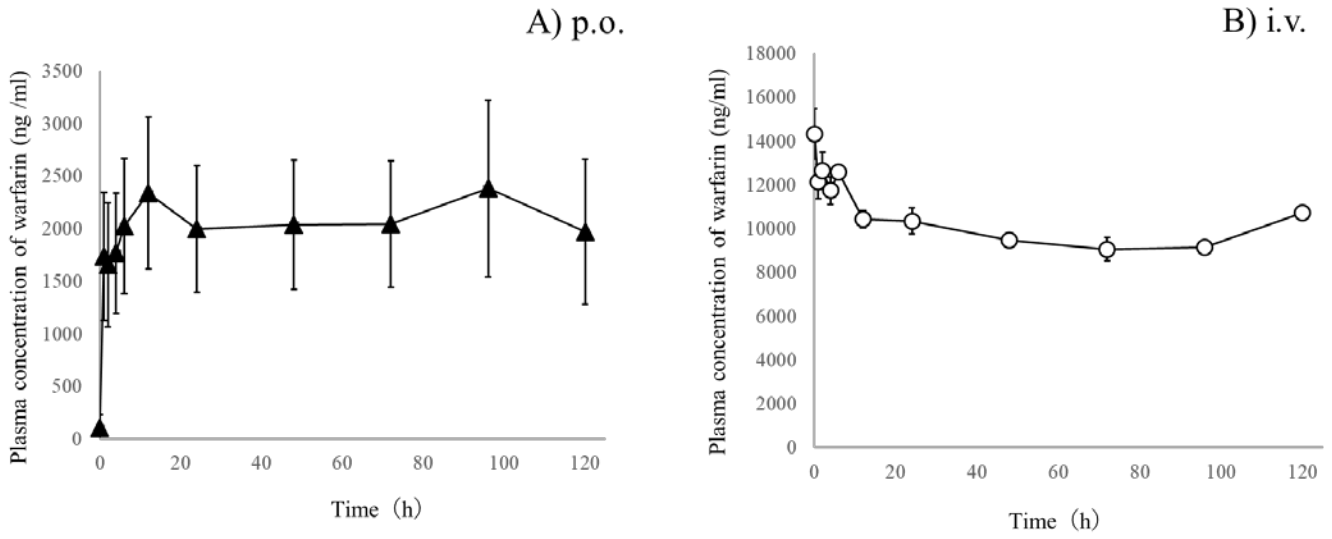
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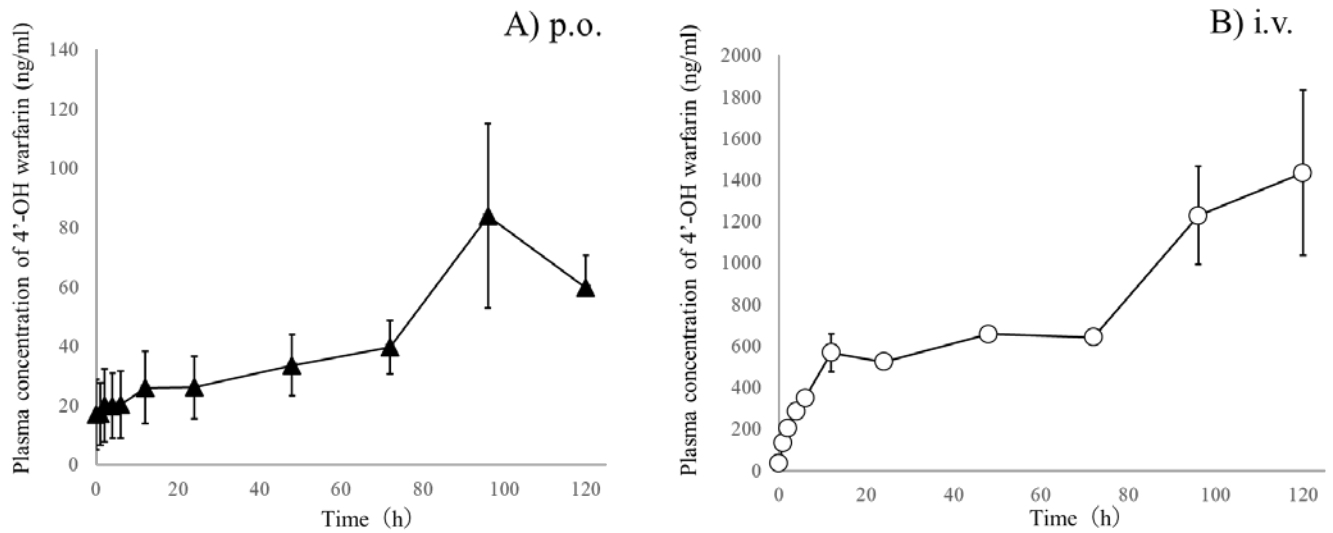
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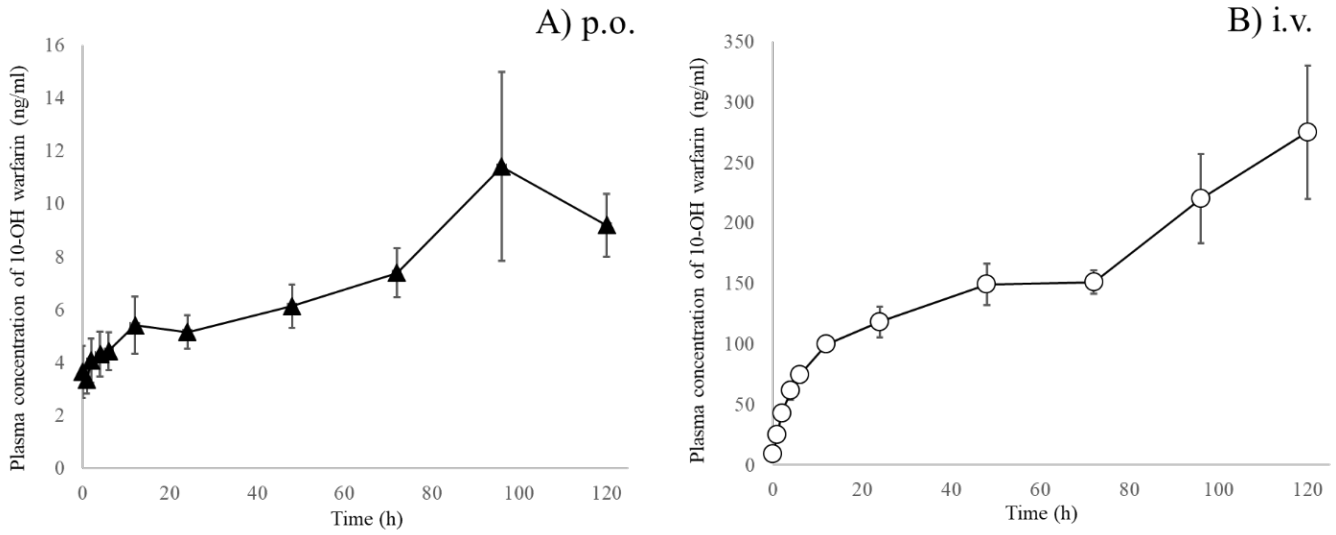
626 **Fig. 1.** Time course of changes in plasma warfarin concentration after oral (A) or intravascular (B)
627 administration of 4 mg/kg warfarin. Blood collection was performed at 5 min(0h) and 1, 2, 4, 6, 12, 24,
628 48, 72, 96, and 120 h after oral administration (p.o.; n = 4) or intravenous administration (i.v.; n = 3).
629 Data are presented as mean (points) ± standard error (error bars).

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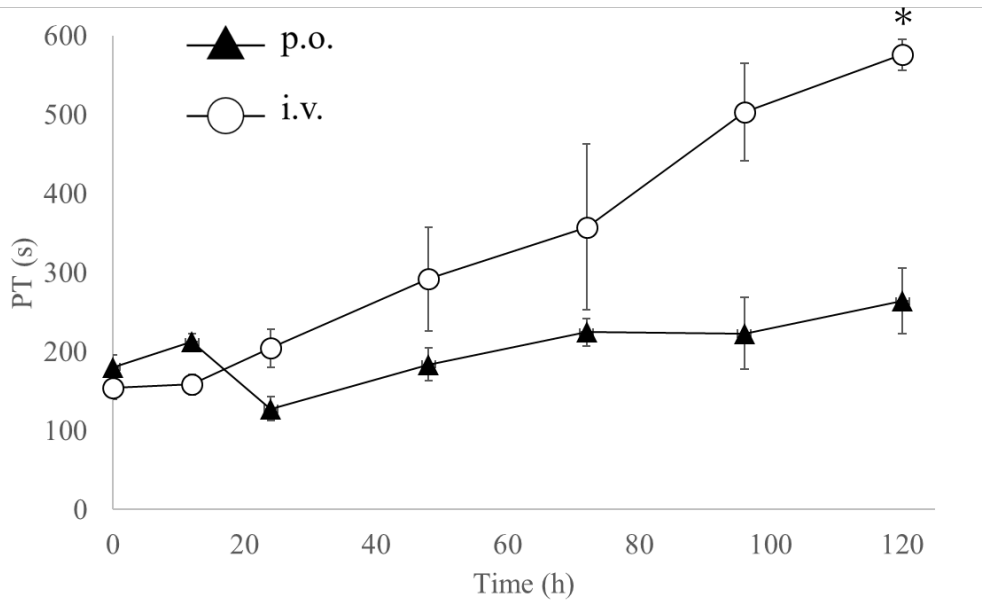


639 **Fig. 2.** Time course of changes in plasma 4'-OH warfarin concentration after oral (A) or intravascular
 640 (B) administration of 4 mg/kg warfarin. Blood collection was performed at 5 min (0h) and 1, 2, 4, 6,
 641 12, 24, 48, 72, 96, and 120 h after oral administration (p.o.; n = 4) or intravenous administration (i.v.; n
 642 = 3). Data are presented as mean (points) ± standard error (error bars).

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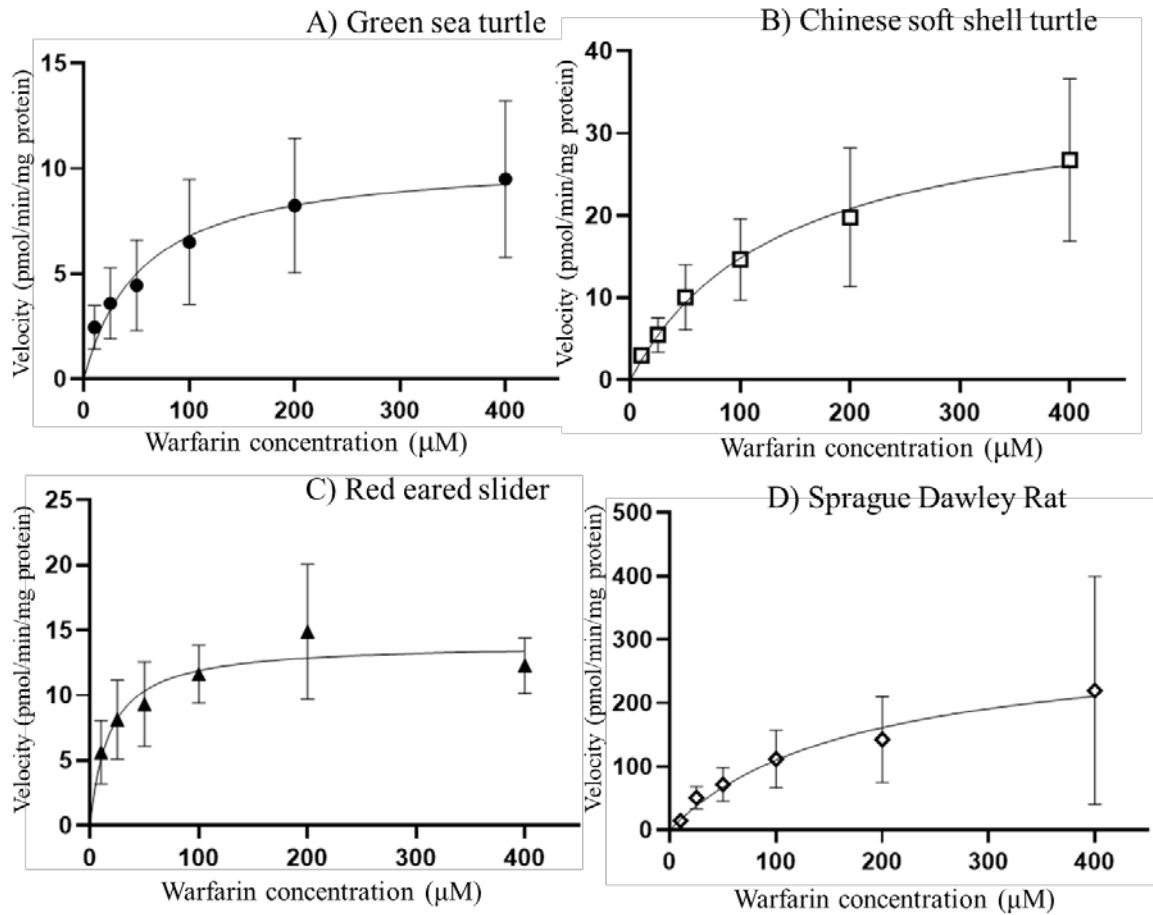
654 **Fig. 3.** Time course of changes in plasma 10-OH warfarin concentration after oral (A) or intravascular
 655 (B) administration of 4 mg/kg warfarin. Blood collection was performed at 5 min(0h) and 1, 2, 4, 6, 12,
 656 24, 48, 72, 96, and 120 h after oral administration (p.o.; n = 4) or intravenous administration (i.v.; n =
 657 3). Data are presented as mean (points) ± standard error (error bars).



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

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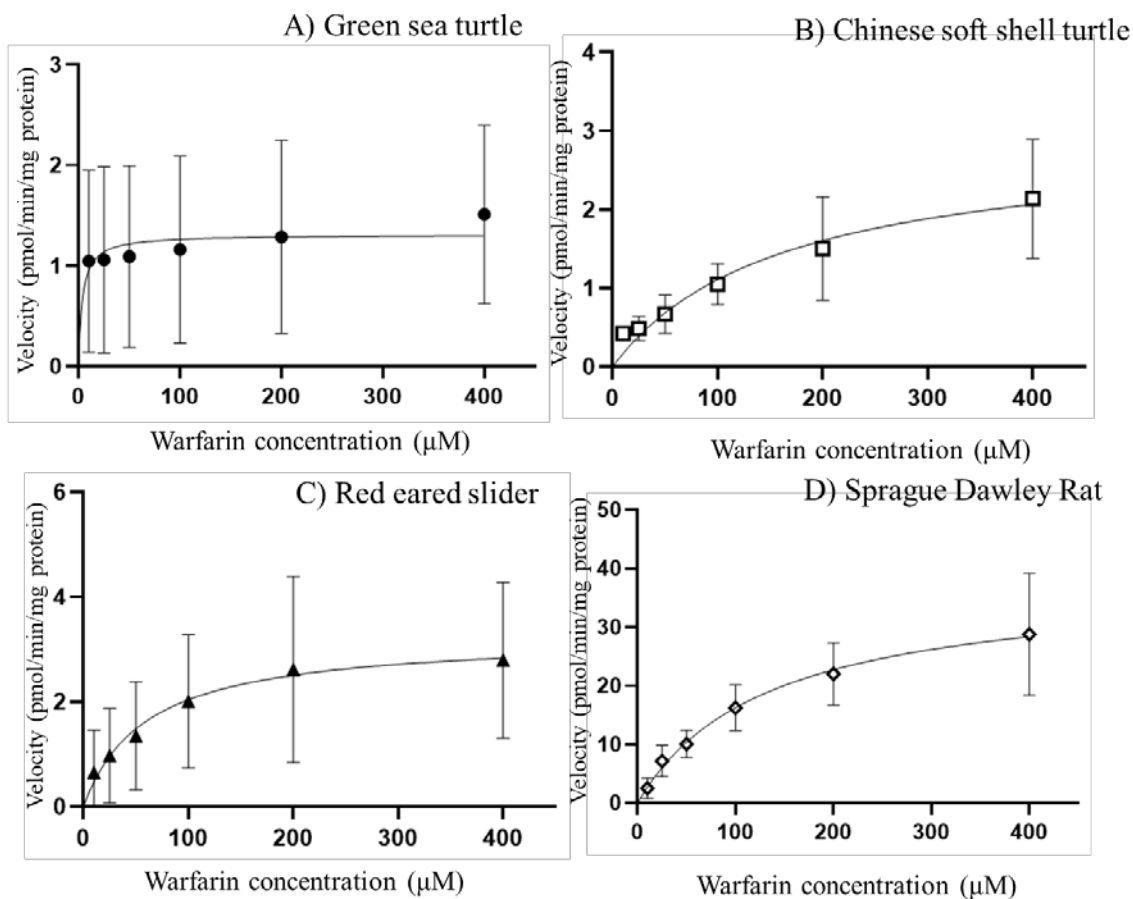
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670 **Fig. 5.** Michaelis–Menten plots of warfarin 4'-hydroxylation in three turtle species (green sea turtle,
 671 Chinese softshell turtle, and red-eared slider) and Sprague Dawley rats. Data are presented as mean
 672 (points) \pm standard error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

673

674



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

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684 **Table 1**685 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	<i>Chelonia mydas</i>	unknow n	< 1 year	2.2 ± 0.14	7	a	<i>In vivo</i> exposure
Green sea turtle	<i>Chelonia mydas</i>	male	adult	98.8 ± 3.7	5	b	<i>In vitro</i> metabolism & VKOR inhibition test
Chinese softshell turtle	<i>Pelodiscus sinensis</i>	male	adult	0.93 ± 0.02	4	c	<i>In vitro</i> metabolism & VKOR inhibition test
Red-eared slider	<i>Trachemys scripta elegans</i>	male	adult	0.52 ± 0.06	5	d	<i>In vitro</i> metabolism & VKOR inhibition test
Sprague Dawley rat	<i>Rattus norvegicus</i>	male	7 weeks	205 ± 5 *	5	e	<i>In vitro</i> metabolism & VKOR inhibition test

686 Body weights are presented as mean ± standard error.

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

688 a: Ogasawara Marine Center (Tokyo, Japan)

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

690 c: Local restaurant (Sapporo, Japan)

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

692 e: Japan SLC (Shizuoka, Japan)

693 **Table 2**

694 Metabolism of warfarin into its hydroxylated forms, as revealed by the kinetic parameters of
 695 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	

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

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706 **Table 3**

707 VKOR activity in green sea turtles

Vmax (pmol/min/ mg protein)	Km (μ M)	Vmax/Km
29.6 \pm 3.1	4.7 \pm 0.7	6.7 \pm 0.7

708 Kinetic parameters of VKOR activity in green sea turtles. The values presented are means \pm standard

709 error (n = 5).

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729 **Table 4**

730 Warfarin IC₅₀

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

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732 Mean ± standard error IC₅₀ (half-maximal inhibitory concentration) values for warfarin. The IC₅₀
733 represents the warfarin concentration that inhibits 50% of VKOR activity. There were no significant
734 differences between any of these species (*p* > 0.05; Steel–Dwass test). S: Sprague Dawley rat, G: Green
735 sea turtle, C: Chinese softshell turtle, R: Red-eared slider

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742 **9. References**

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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~~ 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. 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, K₂HPO₄, KH₂PO₄, 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 μ l 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 $2,000 \times g$ 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 μ l of prepared Neoplastine was mixed with 50 μ l 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 μ l) were added to 15 ml centrifuge tubes with 0.1 M sodium acetate (2 ml), 1 μ M glucuronidated oxazepam (100 μ l, as an internal standard for warfarin and an indicator of deconjugation), 1 μ M phenol-d5-7-hydroxywarfarin (10 μ l, as an internal standard for hydroxywarfarin), and 4,500 units of β -glucuronidase (100 μ l). 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 \times 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 N₂ gas. The residue was dissolved in MeOH (200 μ l).

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 \times g for 20 min was further

centrifuged twice at $100,000 \times g$ 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 activity was linear. Magnesium chloride (3 mM, final concentration), α -Glucose-6-phosphate (G6P) (5 mM, final concentration), and 10, 25, 50, 100, 200, or 400 μ M 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 μ l. Samples were preincubated for 5 min. A 10 μ l mixture of α -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. Turtles are ectothermexothermal 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 K_m and V_{max} 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 N₂ 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 $57.45\% \pm 17.00\%$ ($n = 4$). The recovery rate of warfarin was $108.22\% \pm 31.72\%$. 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 $83.49 \pm 1.64\%$, 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 IC₅₀ 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 \pm 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) \pm 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) \pm standard error (error bars). The final substrate (warfarin) concentration was 400 μ M. 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

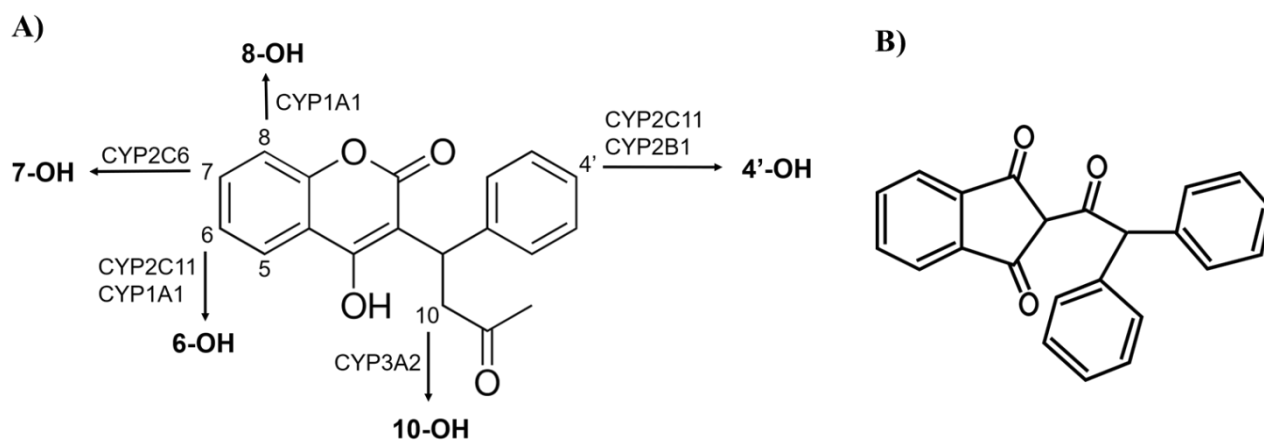


Fig. S1. A) Metabolic pathways of warfarin metabolites in rats. Warfarin is hydroxylated by members of the cytochrome P450 superfamily. Various types of CYP are responsible for hydroxylating warfarin. There are five types of warfarin metabolite: 4'-, 6-, 7-, 8-, and 10-OH warfarin. B) Chemical structure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

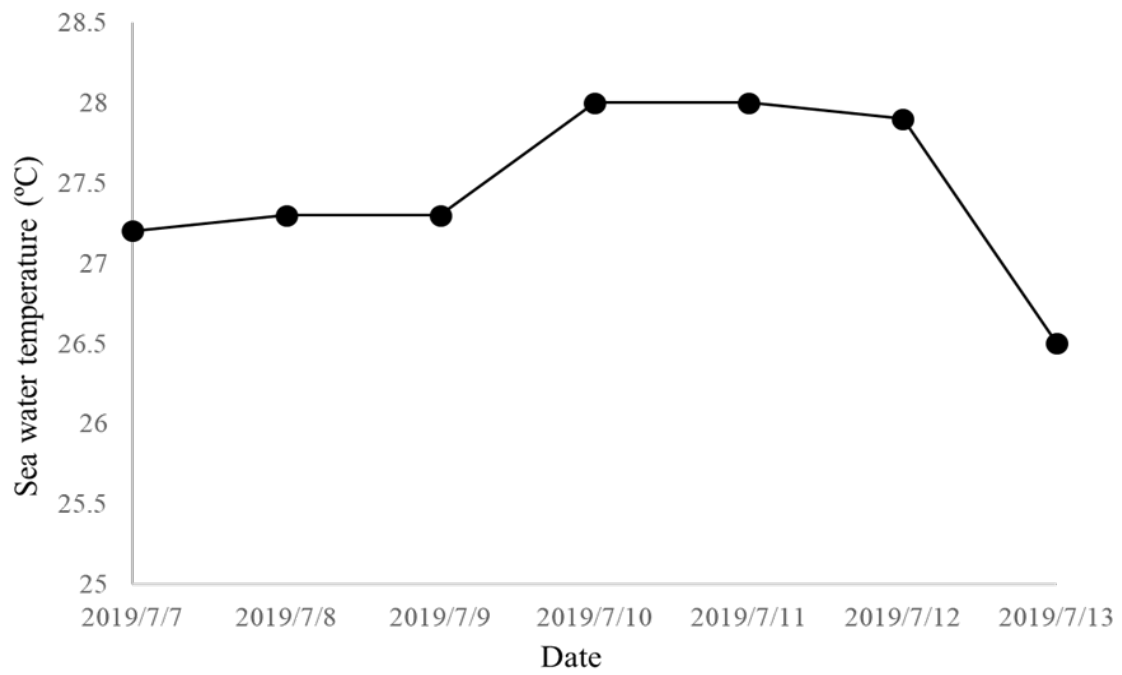


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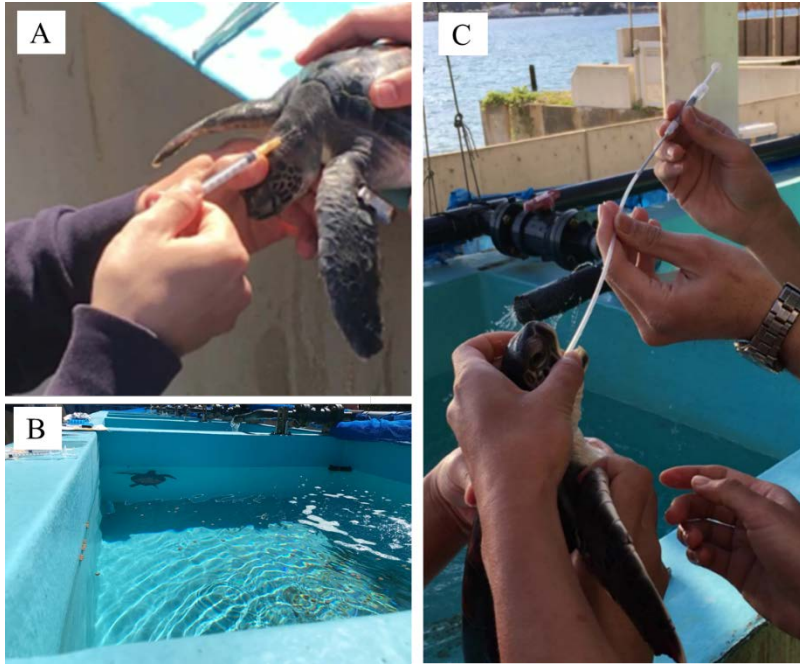


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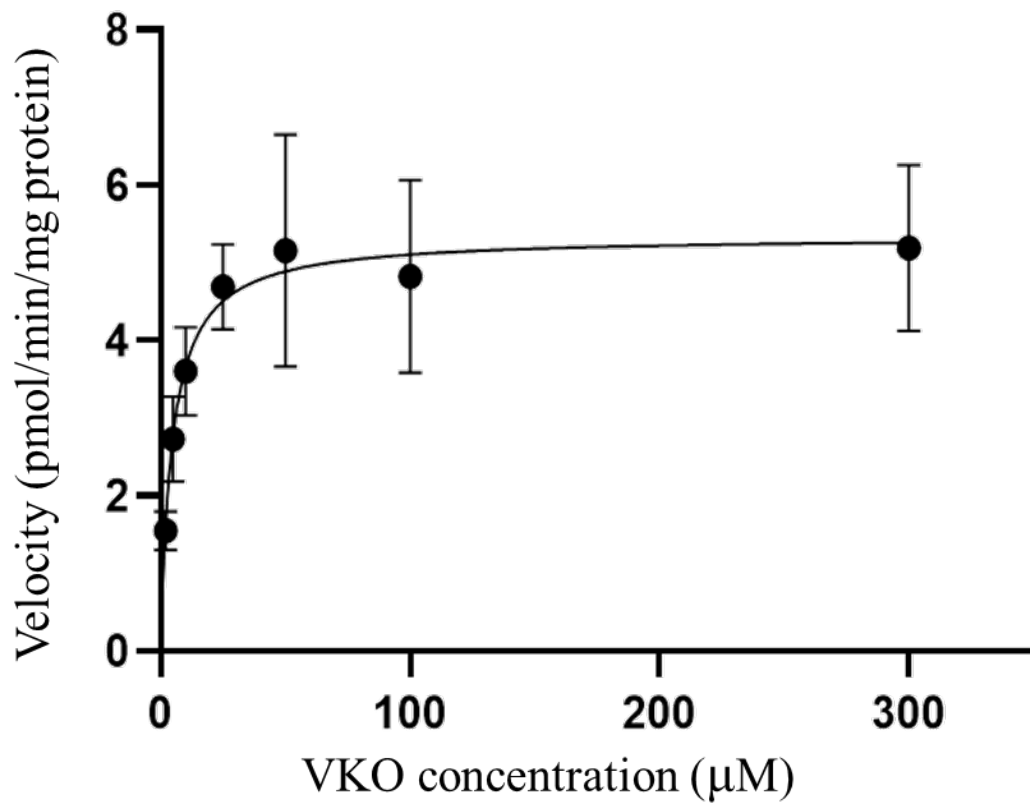


Fig. S4. Michaelis–Menten plot of VKOR activity in green sea turtles. Data are presented as mean (points) \pm standard error (error bars).

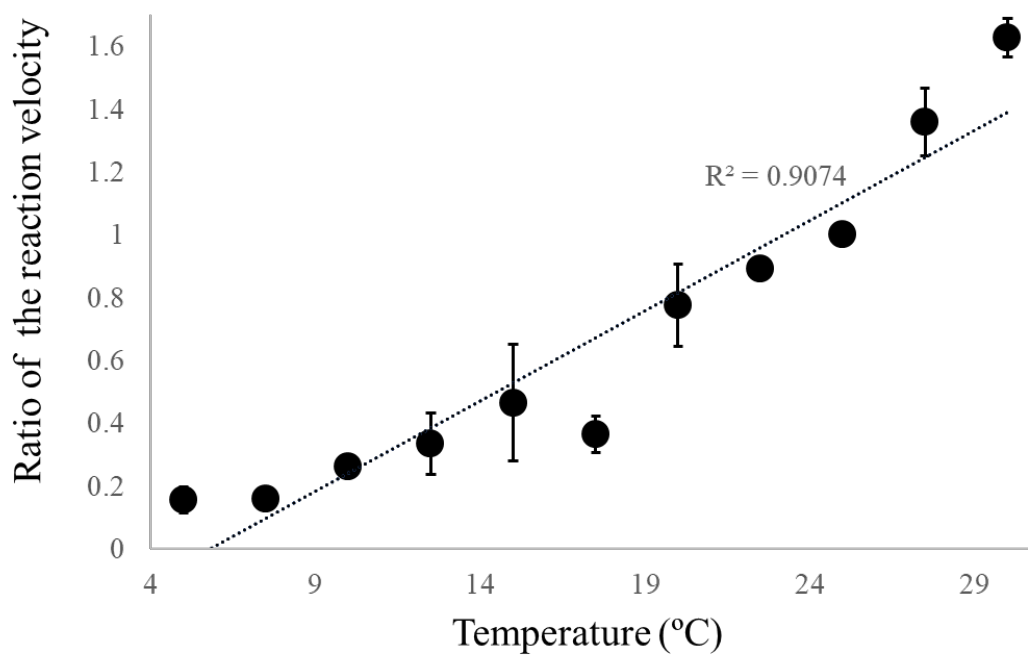


Fig. S5. The relationship between incubation temperature and the rate of conversion of warfarin to 4'-OH warfarin in male Chinese softshell turtles (*Pelodiscus sinensis*) ($n = 2$). Data are presented as mean (points) \pm standard error (error bars). The final substrate (warfarin) concentration was 400 μ M. 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

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