Avian interspecific differences in VKOR activity and inhibition: insights from amino acid sequence and mRNA expression ratio of VKORC1 and VKORC1L1

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ABSTRACT

Worldwide use of anticoagulant rodenticides (ARs) for rodents control has frequently led to secondary poisoning of non-target animals, especially raptors. In order to suggest some factors that may help considering the mechanism of the incidents, this study focused on the avian vitamin K 2, 3-epoxide reductase (VKOR) that is the target protein of ARs. We addressed the interspecific differences in VKOR activity and inhibition related to amino acid sequence and mRNA expression of VKORC1 and VKORC1-like1 (VKORC1L1). Poultry have been considered to be more tolerant to ARs than mammals. However, VKOR activity of owls, hawks, falcon and surprisingly, canaries, was lower and inhibited by warfarin more easily than that of chickens and turkeys. The amino acid sequence of VKORC1 and VKORC1L1 implied that the value of Ki for VKOR activity to ARs could depend on the amino acid at position 140 in the TYX warfarin-binding motif in VKORC1, and other amino acid mutations in VKORC1L1. The mRNA expression ratio of VKORC1:VKORC1L1 differed between turkey (8:1) and chicken (2:3) liver. VKORC1L1 has been reported to be resistant to warfarin compared to VKORC1. Hence, both the Ki of specific VKORC1 and VKORC1L1, and the mRNA expression ratio would cause avian interspecific difference of the VKOR inhibition. Our study also suggested the high inhibition of VKOR activities in raptors and surprisingly that in canaries as well. These factors are the most likely to contribute to the high sensitivity to ARs found in raptors.

Keywords: anticoagulant rodenticides, VKORC1, VKORC1L1, raptors,

INTRODUCTION

Worldwide use of anticoagulant rodenticides (ARs) for vertebrate pest control has led to the unintentional exposure of non-target animals, especially raptors, to these poisons (López-Perea et al., 2018). Exposure pathways of ARs to raptors have been presumed to be prey on target rodents or non-target animals. Rattner et al. (2018a) summarize the median lethal dose (LD_{50}) for anticoagulant rodenticides (ARs) among animals. Avian species seem more tolerant to ARs than mammals. However, the LD_{50} for the first-generation AR diphacinone in the American kestrel (*Falco sparverius*) is lower than in poultry (Rattner et al., 2011), and a comparative risk model found that second-generation ARs pose a far greater risk to predatory and scavenging birds (Erickson et al., 2004; Anderson et al., 2011). Considering both the low values of LD_{50} in raptors and the frequent poisoning of non-target wild birds, especially raptors, implies raptors have different susceptibility to ARs than poultry.

Interspecific difference in sensitivity to ARs could be caused by differences in the both AR metabolism by cytochrome P450 (CYP) and the Vitamin K 2, 3-epoxide reductase (VKOR) inhibition, which is the pharmacological target of AR. However, those data are limited to very few avian species. Watanabe et al. (2010) used warfarin as a model compound of ARs and reported that owls in particular showed very low CYP-dependent warfarin metabolic activity compared with other avian species and rats. Rattner et al. (2014) showed that the eliminated half-life of diphacinone in the liver was longer in eastern screech-owls (*Megascops asio*) than in mammals. These studies imply that owls may possess a low ability to detoxify ARs. On the other hand, there are few studies on VKOR characterization in raptors. The VKOR IC50 of several ARs in hepatic microsomes of the American kestrel has been described (Rattner et al., 2018b), i.e., brodifacoum (0.22 μM), and preliminary warfarin (177 μM) and chlorophacinone (5.1 μM). Brodifacoum was reported to have similar inhibition efficiency for VKOR containing hepatic microsomes to mammals (IC50 of 0.15-0.26 μM in mammals).

VKOR catalyzes the reduction of vitamin K 2, 3-epoxide (VKO) to vitamin K

quinone (vitamin K) (Fig. S1). This reaction is inhibited by ARs such as warfarin. Vitamin K is reduced to vitamin K hydroquinone (VKH_2) by vitamin K quinone reductase (VKR). VKH_2 is a cofactor for the γ-glutamyl carboxylation of glutamate (Glu) in vitamin K dependent proteins (VKDPs). Some of the most studied VKDPs are clotting factors II, VII, IX, and X in mammals (Ferland, 1998). Although it was thought that avian clotting factors were different from that of mammals (Walz et al., 1975; Frost et al., 1999), recent studies show that avian plasma possesses functional coagulation factors (Thomson et al., 2002), and that avian prothrombin times (chicken, 9.7 s – 27 s) are similar to those of mammals (human, 14 s – 17 s) (Frost et al., 1999; Webster 2009). The inhibiting VKOR by ARs impedes carboxylation of blood clotting factors which can result in hemorrhage in both birds and mammals (Cain et al., 1998; Rattner et al., 2012; Rattner et al., 2014; Rattner et al., 2015).

There are two paralogous multisubunit membrane protein complexes that perform VKOR activity, VKOR complex 1 (VKORC1) and VKORC1-like1 (VKORC1L1) (Tie and Stafford, 2016). In mammals, the focus has principally been on VKORC1. Spohn et al. (2009) demonstrated that VKORC1 knockout caused early postnatal lethality due to severe bleeding in mice. Hammed et al. (2013) and Caspers et al. (2015) reported that the mRNA expression of VKORC1 was over 10-fold higher than that of VKORC1L1 in the liver of rats and mice. Therefore, VKORC1 has been considered as the main protein supporting VKOR activity in liver of mammals. However, VKORC1L1 was described to be involved in VKOR activity in some extrahepatic tissues (Hammed et al., 2013). Moreover, warfarin inhibition constant K_i for rat VKORC1L1 was reported to be higher than that for rat VKORC1. The authors demonstrated that mRNA expression of VKORC1L1 was higher than that of VKORC1 in rat testis, and the VKOR activity of the testis was not inhibited as easily by warfarin compared to VKOR activity of the liver. These facts imply that a high mRNA expression of VKORC1L1, which has a high K_i, caused warfarin resistance. Hence, warfarin susceptibility could be caused by both the mRNA expression ratio, and the individual K_i of VKORC1 and

VKORC1L1.

Although the warfarin-binding site of VKOR has not been clearly identified, amino acids at the 138 to 140 positions in the rat VKORC1 “TYX motif” may be the warfarin-binding site. In rats and mice, tyrosine 139 mutations in VKORC1 exhibit high resistance to warfarin compared to other mutations (Lasseur et al., 2006a; Lasseur et al., 2006b; Rost et al., 2009). The Thr-Tyr-Ala motif also exists in NAD(P)H quinone oxidoreductase (NQOR) that is sensitive to warfarin (Rost et al., 2005). The TYX warfarin-binding site could be important to the K_i for VKOR activity in response to ARs.

There are few studies on avian VKOR. It was reported that the V_{max} (maximum velocity) of VKOR activity in chickens and ostriches was 3- to 7-fold lower than that of rats, and K_i for VKOR activity after warfarin treatment was 17- to 40-fold higher in chickens than in ostriches or rats (Watanabe et al., 2010). These facts were the first to indicate low VKOR activity in poultry compared to mammals and the high warfarin resistance of chickens. Nevertheless, it was not enough to elucidate broad avian interspecific differences in sensitivity to ARs, especially the high susceptibility to ARs found in raptors.

This study addressed avian interspecific differences in VKOR activity and sensitivity to warfarin, one of the most common ARs in the world. The cause of avian interspecific differences in VKOR activity and sensitivity were discussed by comparing amino acid sequence and mRNA expression of VKORC1 and VKORC1L1 among avian species, including raptors. The aim of this study was to reveal the factors contributing to the high sensitivity to ARs found in raptors, which are frequently reported as non-target poisoning instances.

MATERIALS AND METHODS

Chemicals

HEPES was purchased from Dojindo Laboratories (Kumamoto, Japan). Vitamin K_1 was from Kanto Chemicals (Tokyo, Japan). Diethyl ether and RNA*later*® were from Sigma-Aldrich Co. (St. Louis, MO). Racemic warfarin, dithiothreitol (DTT), isopropyl alcohol, hexane, ethanol, methanol, H_2O_2, K_2HPO_4, KH_2PO_4, Na_2CO_3, and NaOH were purchased from Wako Pure Chemical Industries (Osaka, Japan). Isoflurane was purchased from DS Pharma Animal Health Co. (Osaka, Japan).

Animals

All avian species including raptors used in this study are shown in Table 1. Chickens (13 months old) were provided from the farm at Hokkaido University (Sapporo, Japan). Turkeys (20 months old) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Canaries (six months old) were purchased from a local pet shop (Sapporo, Japan). These three species were included in this study because the genome information of both VKORC1 and VKORC1L1 are available. Turkeys and canaries were acclimatized to the environment at a constant temperature (22°C ± 1°C) with a 12:12 h light:dark cycle, and given food and water *ad libitum* for 1 week before commencement of the experiment. Chickens, turkeys and canaries were anesthetized using isoflurane and euthanized by CO_2. After euthanasia, nine different tissues were dissected, including liver, pancreas, spleen, testis or ovary, kidney, lung, heart, brain, and muscle. The excised tissues were cut into small pieces and stored in RNA*later*® (Sigma-Aldrich) at –20°C after an overnight incubation at 4°C. The rests of the tissues were immediately frozen in liquid nitrogen and stored at –80°C until use.

Livers of raptors were provided by the Institute for Raptor Biomedicine Japan (Kushiro, Japan), and Sapporo Maruyama Zoo (Sapporo, Japan). After the raptors were dead due to illness or traffic accidents, some raptor individuals were dissected immediately, while dead body of some individuals were kept at 4°C in a few days, then

158 dissected. After dissection, liver samples were immediately frozen in liquid nitrogen and
159 stored at –80°C until use.
160 All experiments using animals were performed under the supervision and with
161 the approval of the Institutional Animal Care and Use Committee of Hokkaido University,
162 Japan (approval number 14-0119).
163
164 *Preparation of liver microsomes*
165 Livers were taken from animals and liver microsomes were prepared as
166 described previously (Watanabe et al., 2010). Because the livers of canaries were very
167 small, two pools were made from six livers for the preparation of microsomes. For other
168 species, a microsome was made from an individual. Briefly, the livers were homogenized
169 with three times their volume of potassium phosphate buffer (KPB, 0.1M, pH7.4). The
170 homogenates were centrifuged at 9000 ×g at 4°C for 20 min. The supernatant was
171 decanted to an ultracentrifugation tube and centrifuged at 105000 ×g at 4°C for 60 min.
172 The pellet was homogenized in KPB in ice and then centrifuged again at 105000 ×g at
173 4°C for 60 min for washing. The resultant microsomal pellets were homogenized in KPB
174 again. The suspensions were transferred to 1.5 mL tubes and stored at −80°C until use.
175 The protein concentration of hepatic microsomes was measured with the BCA protein
176 assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacture's
177 instruction.
178
179 *Preparation of VKO*
180 VKO was produced as previously described (Tishler et al., 1940). Briefly, 100
181 mg of vitamin K_1 was dissolved in 5 mL of ethanol and pre-incubated at 75°C for 5 min.
182 1 mL of 30% hydrogen peroxide and 250 μL of water containing 100 mg of sodium
183 carbonate were added to the vitamin K solution and the mixture was kept at 75°C for 15
184 min. The mixture was cooled, diluted with 10 mL of water and 40 mL of diethyl ether,
185 and centrifuged at 1000 ×g for 10 min. The supernatant was evaporated under nitrogen

and dissolved in methanol. The solution was refined by high-pressure liquid chromatography (HPLC) using the following components: a PU-980 pump (Jasco, Tokyo, Japan); an Inertsil PREP-ODS column, 30.0 × 250 mm (GL Science Inc., Tokyo, Japan); a Mightysil, RP-18GP Aqua guard column (Kanto Chemical Co. Inc., Tokyo, Japan); and a 4.6 × 5 mm, 5 μm SPD-6AV detector (SHIMADZU, Kyoto, Japan). The detection wavelength was 270 nm, the flow rate 10.0 ml/min, and the mobile phase 3% double distilled water (DDW) in methanol. The refined solution was evaporated under nitrogen and dissolved in ethanol. The VKO was kept at 4°C and shielded from light until use.

The concentrations of VKO and vitamin K_1 were determined spectrophotometrically using a molar absorption coefficient of 30,800 M^{-1} cm^{-1} at 266 nm and of 18,900 M^{-1} cm^{-1} at 249 nm each (Wallin and Martin, 1987).

VKOR activity assay

VKOR activity was assayed in duplicate for each sample as described previously with slight modifications (Watanabe et al., 2010; Hammed et al., 2013). The reaction mixture (500 μL, total volume) contained microsomes (1.0 mg/ml, final concentration), HEPES buffer (pH 7.4, 0.1 M), and VKO (12.5, 100 or 400 μM). After 5 min pre-incubation, the reaction was started by the addition of 2 mM DTT solution. The reaction was performed for 5 min and stopped by adding 1 mL of an iced 1:1 isopropyl alcohol/hexane solution.

The temperatures for pre-incubation and reaction were 41.5°C for turkeys, 42°C for chickens, 38.5°C for canaries, 40.5°C for a peregrine falcon, sparrowhawks, goshawk, Steller's sea eagle and white-tailed eagle, and 39.5°C for snowy owls and great horned owls (McNab 1966; Richards 1971; Siegfried et al., 1975; Chaplin et al., 1984; Herrero and Barja, 1998).

To perform the liquid-liquid extraction of vitamin K, 2 mL of 1:1 isopropyl alcohol/hexane solution and 1 mL HEPES buffer were added. After centrifugation at 750 ×g for 10 min, the organic layer was collected and dried under nitrogen. The dry residue

was dissolved in 80 μL of methanol, and the reaction product was analyzed by HPLC coupled with UV-VIS detector quantified at 270 nm (Pump: LC-20AB, Detector: SPD-20A; SHIMADZU, Kyoto, Japan). Separation was achieved on an Inertsil ODS-3, 2.1 × 150 mm, 5 μm analytical column (GL Sciences, Kyoto, Japan) run at 40°C at 0.5 mL/min in 99.8% methanol. Vitamin K concentration in all samples except for *Haliaeetus* (Steller's sea eagle and white-tailed eagle) was higher than the limit of quantitation (0.0378 μM).

Inhibition of VKOR activity

The assays were done using the same procedure as for the VKOR activity assay as described above. The reaction was performed at concentrations of 100 μM of substrate (VKO) and 1 or 10 μM of warfarin-sodium.

Total RNA extraction and cDNA synthesis

Total RNA was extracted from ten different tissues (liver, pancreas, spleen, testis or ovary, kidney, lung, heart, brain and muscle) of chickens and turkeys, and the livers of night heron, snowy owl, and great horned owl, using NucleoSpin® RNA II (TAKARA BIO INC., Tokyo, Japan). The purity and quantity of RNA were determined by electrophoresis as well as spectrophotometry using NanoDrop ND-1000 (Thermo Scientific, DE). $A_{260/280}$ and $A_{260/230}$ were generally ≥2. Total RNA (10 μg) was reverse transcribed using ReverTra Ace (TOYOBO, Osaka, Japan) in a final volume of 100 μl, according to manufacturer's instructions.

Partial cloning of VKORC1L1

VKORC1L1 of night heron, snowy owl and great horned owl were cloned in this study. The cDNA was amplified by polymerase chain reaction (PCR) using specific primers shown in Table S1. The PCR was performed using *Ex Taq*® (Takara, Tokyo, Japan). The PCR cycle program was on one cycle of 30 s at 94°C, 40 cycles of 30 s at

94°C, 30 s at 56.8°C, and 30 s at 72°C, with one cycle of final extension for 1 min at 72°C.

Plasmids were constructed with the PCR products and pCR2.1-TOPO vector using a TOPO TA Cloning Kit (Invitrogen, CA) and transformed into DH5α-competent cells (TOYOBO, Osaka, Japan). Plasmids were purified using a plasmid miniprep spin kit (Qiagen, Tokyo, Japan). Inserts were sequenced using a BigDye Terminator version 1.1 (Applied Biosystems, Foster City, CA). Ethanol precipitation was performed after the amplification reaction, and the plasmid sequence was analyzed by an automated DNA sequencer, ABI Prism 310 Genetic Analyzer (Thermo Fisher Scientific Inc., Kanagawa, Japan), following the manufacturer's instructions.

Amino acid sequence alignment of VKORC1 and VKORC1L1

The VKORC1 and VKORC1L1 genes of animals including various avian species were retrieved using an NCBI BLAST search (https://blast.ncbi.nlm.nih.gov/Blast.cgi?CMD=Web&PAGE_TYPE=BlastHome; Table S2). Amino acid sequences were aligned by MUSCLE (Edgar 2004).

Phylogenic analysis

The phylogenetic relationship of VKORC1 and VKORC1L1 was inferred by using the Maximum Likelihood method based on the Le_Gascuel_2008 model (Le and Gascuel, 2008). The model was selected based on Akaike information criterion with a correction for finite sample sizes (AICc). The bootstrap consensus tree inferred from 500 replicates (Felsenstein 1985) [32]. Branches with poor bootstrap values (less than 60%) are collapsed. Initial tree(s) for the heuristic search were constructed by the Neighbor-Joining method with a JTT model. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 2.8254)). The rate variation model allowed for some sites to be evolutionarily invariable ([+I], 8.9717% sites). The analysis involved 42 amino acid sequences and 93 positions. Only positions

with more than 85% site coverage were analyzed. Phylogenetic analyses were performed by Molecular Evolutionary Genetics Analysis (MEGA) ver. 6.06 (Tamura et al., 2013).

Plasmid constructions for quantitative real-time PCR

The cDNA of chickens and turkeys was amplified by PCR using specific primers for VKORC1, VKORC1L1, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and actin beta (β-actin) shown in Table S3. Because VKORC1 of chickens and turkeys could not be cloned using the same primers as for quantitative real-time PCR, VKORC1 was cloned using the alternative primers. The PCR was performed using SapphireAmp® (Takara, Tokyo, Japan). The PCR cycle program was on one cycle of 30 s at 94°C, 40 cycles of 5 s at 98°C, 5 s at 63°C, and 5 s at 72°C, with one cycle of final extension for 1 min at 72°C.

Plasmids were constructed with the PCR products and pCR2.1-TOPO vector using a TOPO TA Cloning Kit (Invitrogen). All sequences inserted into the plasmids include an amplicon of the quantitative real-time PCR products.

Quantitative real-time PCR

Gene-specific quantitative real-time PCR primers (Table S3) were synthesized by Sigma- Aldrich (Tokyo, Japan). The efficiency of all primers was 89%–107%. Quantitative real-time PCR (qPCR) was performed with the StepOnePlus Real-Time PCR system (Applied Biosystems, Foster City, CA). The 10 µl PCR reaction mixtures consisted of the Fast SYBR Green Master Mix (Applied Biosystems), forward and reverse primers (200 nM each), and cDNA derived from 80 ng of total RNA. Plasmids containing each amplicon were used for the calibration curves. All samples, including cDNA derived from the tissues of chickens and turkeys and the plasmid standards, were analyzed in duplicate using the following protocol: 95°C for 20 s followed by 40 cycles of 95°C for 3 s and 60°C for 30 s. At the end of each PCR run, melt curve analysis was performed in the range of 60°C–95°C. PCR products were confirmed as single fragments

298 by electrophoresis and direct or plasmid sequencing methods. For the negative control,
299 DDW was added in the reaction mixture instead of cDNA, and confirmed no
300 contamination

301 Both the GAPDH and the β-actin tested in this study were not appropriate as
302 housekeeping genes among various tissues, since the differences of Ct values among
303 various tissues varied 5-10, resulting in the wide variation of copy numbers (2^5–2^{10}
304 copies). In addition, because appropriate housekeeping were not found in any other avian
305 genes (Olias et al., 2014), mRNA expression of VKORC1 and VKORC1L1 were not
306 compensated and shown as “copy number/ng total RNA” calculated from the standard
307 curve with plasmids.

308

309 *Statistical analysis*

310 For the comparison of VKOR activity and inhibition assays among avian
311 species, significant difference among avian groups were analyzed using a Steel-Dwass
312 test because the data were not parametric. All the statistical analysis was performed with
313 a significance level of $p < 0.05$, using JMP software (version 12.0; SAS Institute, Cary,
314 NC). Because of the small sample size, goshawk and sparrowhawks, and snowy owls and
315 great horned owls were grouped for statistical testing *(Accipiter* and *Bubo*, respectively).

RESULTS

Comparison of VKOR activity among avian species

Results of VKOR activity among eight avian species are shown in Fig. S2 and Table 2. At every concentration of VKO (12.5, 100, and 400 μM), there was no significant difference between male (78 ± 28, 124 ± 50, and 153 ± 57 pmol/min/mg protein respectively, n=3) and female (49 ± 31, 97 ± 66, and 103 ± 65 pmol/min/mg protein respectively, n=3) chickens. Therefore, both sexes from the same species were analyzed together in other avian samples.

VKOR activities of raptors and canaries were lower than that of turkeys and chickens (Table 2, Fig. S2). At 400 μM VKO, VKOR activities were following orders; turkeys (270 ± 17 pmol/min/mg protein, n=3), male chickens (153 ± 57 pmol/min/mg protein, n=3), female chickens (103 ± 65 pmol/min/mg protein, n=3), *Bubo* (snowy owls and great horned owls; 83 ± 21 pmol/min/mg protein, n=4), and *Accipiter* (goshawk and sparrowhawks; 45 ± 5 pmol/min/mg protein, n=3). The VKOR activity of peregrine falcon was only 14 pmol/min/mg protein (n=1). That of canaries was 85 pmol/min/mg protein (2 pools). VKOR activity in raptors ranged to a small fraction (5.2–81%) of that observed in turkey and chicken hepatic microsomes. VKOR activity in peregrine falcon accounted for 5.2% of that in turkeys, and VKOR activity in *Bubo* accounted for 81% of that in female chickens. VKOR activities of *Haliaeetus* (Steller's sea eagle and white-tailed eagle) were undetectable.

Comparison of VKOR activity inhibited by warfarin among avian species

Fig. 1A shows VKOR activity without warfarin, and VKOR activity with 1 or 10 μM warfarin incubation at 100 μM of VKO. With 1 μM warfarin, VKOR inhibition did not occur in chickens and turkeys. However, VKOR inhibition was found in other avian species. With 10 μM warfarin, inhibition of VKOR activity occurred in every avian species. For male chickens (n=3), VKOR activity without warfarin, with 1 μM warfarin, and with 10 μM warfarin were 124 ± 50, 132 ± 53, and 83 ± 27 pmol/min/mg protein,

344 respectively. For female chickens (n=3), VKOR activity without warfarin, with 1 μM
345 warfarin, and with 10 μM warfarin were 97 ± 66, 104 ± 71, and 72 ± 44 pmol/min/mg
346 protein, respectively. For turkeys (n=3), those were 222 ± 36, 207 ± 30, and 117 ± 24
347 pmol/min/mg protein, respectively. For a goshawk, those were 38, 29, and 26
348 pmol/min/mg protein, respectively. For sparrowhawks (n=2), those were 36, 29, and 20
349 pmol/min/mg protein, respectively. For snowy owls (n=2), those were 79, 56, and 35
350 pmol/min/mg protein, respectively. For great horned owls (n=2), those were 61, 52, and
351 19 pmol/min/mg protein, respectively. For a peregrine falcon, those were 14, 12, and 9
352 pmol/min/mg protein, respectively. For canaries (2 pools), those were 75, 33, and 9
353 pmol/min/mg protein, respectively.
354 Fig. 1B shows the percentage of VKOR activity with 1 or 10 μM warfarin
355 incubation compared to VKOR activity of untreated microsomes. For male and female
356 chickens, and turkeys, 1 μM warfarin inhibited control activity by 106 ± 4.6% and 106 ±
357 16%, and 93 ± 1.7%, respectively. For *Accipiter* (a goshawk and sparrowhawks) and *Bubo*
358 (snowy owls and great horned owls), 1 μM warfarin inhibited control activity by 80 ±
359 13% and 79 ± 11%, respectively. 1 μM warfarin inhibited control activity for *Bubo*
360 (snowy owls and great horned owls) compared to male and female chickens. For a
361 peregrine falcon and canaries, 1 μM warfarin inhibited control activity by 85% and 45%,
362 respectively.
363 For male and female chickens, and turkeys, 10 μM warfarin inhibited control
364 activity by 69 ± 8.8%, 75 ± 16%, and 53 ± 2.2%, respectively. For *Accipiter* (a goshawk
365 and sparrowhawks) and *Bubo* (snowy owls and great horned owls), 10 μM warfarin
366 inhibited control activity by 60 ± 13% and 38 ± 15%, respectively. 10 μM warfarin
367 inhibited control activity for *Bubo* (snowy owls and great horned owls) compared to male
368 and female chickens. For a peregrine falcon and canaries, 10 μM warfarin inhibited
369 control activity by 62% and 12%, respectively.
370
371 *Amino acid sequence alignment of VKORC1 and VKORC1L1 in avian species*

The nucleotide sequence of VKORC1 has been defined for only nine avian species in the NCBI database, despite the fact that it has been defined for more than 500 mammals. The nucleotide sequence of raptor VKORC1 has not been clarified. Fig. S3 describes the phylogenetic tree of the VKOR protein. Fig. S4-A shows the amino acid sequence alignment of VKORC1 for nine avian species (turkey, brown roatelo, chicken, ostrich, canary, hummingbird, sandgrouse, crested ibis and emperor penguin) and six other animals (human, rat, mouse, turtle, frog and fugu). The CXXC motif, which is supposed to be the active site of the VKORC1 (Rost et al., 2005; Wajih et al., 2005; Quan et al., 2007), had the following variations: CIVC (human, rat and mouse); CLVC (turkey, brown roatelo, chicken, ostrich, canary, crested ibis, emperor penguin and turtle); CPVC (hummingbird); CVIC (sandgrouse and frog); and CMVC (fugu).

Among avian species, there were three types variations at 68th, 76th, and 143rd amino acid, respectively. The 68th amino acid mutation causes warfarin resistance in human (Hodroge et al., 2012). The 76th amino acid mutation causes warfarin resistance in rats (Tanaka et al., 2012). The 143rd amino acid mutation causes high VKOR activity in rats (Rost et al., 2009). There were two variations among avian species at the 141st amino acid, whose mutation causes low VKOR activity in rats (Rost et al., 2009). There were five variations among avian species at the 140th amino acid in the warfarin-binding site: alanine (turkey and brown roatelo); valine (chicken); glycine (ostrich, canary and hummingbird); isoleucine (sandgrouse); and leucine (crested ibis and emperor penguin).

The nucleotide sequence of VKORC1L1 has been defined for more than 100 avian species and more than 200 mammals in the NCBI database. Fig. S4-B shows the amino acid sequence alignment of VKORC1L1 of seven raptors, ten other avian species, and six other animals. The CXXC motif had the only three variations: CIIC (human, rat, mouse and turtle); CIVC (avian species); and CVIC (frog and fugu).

Few studies have reported the amino acid mutations of VKORC1L1 that contribute to VKOR activity. Hünerberg (2009) used HEK cells expressing recombinant human VKORC1L1, and reported that 36th or 65th amino acid mutations caused warfarin

400 resistance of VKOR activity, and 135th or 146th amino acid mutations caused low VKOR
401 activity. There was no diversity among avian species in the present study at 36th, 65th,
402 135th, or 146th amino acid. However, the 36th amino acid of avian species was different
403 to that of mammals (leucine in avian and valine in mammalian species; Fig S4-B). In the
404 warfarin-binding site, the 147th amino acid of avian species was also different to that of
405 mammals (leucine in avian and valine in mammalian species).
406
407 *Distribution of VKORC1 and VKORC1L1 mRNA in tissues of turkey and chicken*
408 Fig. 2A shows the patterns of the mRNA expression of VKORC1 and
409 VKORC1L1 among nine different tissues from male turkeys. VKORC1 expression in
410 liver was the highest of all tissues (i.e. 4-fold higher than in kidney and testis, 7-fold
411 higher than in lung, 9-fold higher than in spleen, 11-fold higher than in brain, 15-fold
412 higher than in muscle and pancreas, and 16-fold higher than in heart). VKORC1 was also
413 expressed at higher levels than VKORC1L1 in six additional tissues. The expression ratio
414 of VKORC1:VKORC1L1 was 8:1 in liver, 4:1 in pancreas, 3:1 in kidney, 3:2 in spleen,
415 5:4 in muscle and lung, 1:1 in heart and testis, and 2:3 in brain (Fig. 2B).
416 Fig. 3A shows the patterns of the mRNA expression of VKORC1 and
417 VKORC1L1 among ten different tissues from male and female chickens. There was no
418 significant difference in the mRNA expression levels between male and female chickens.
419 VKORC1 expression in ovaries was 2-fold higher than in liver. VKORC1 expression in
420 the other tissues was lower than in liver (i.e., 2-fold lower in lung, spleen and brain, 3-
421 fold lower in kidney, 5-fold lower in testis and heart, 9-fold lower in muscle, and 23-fold
422 lower in pancreas). VKORC1L1 was expressed at higher levels than VKORC1 in all
423 tissues except for spleen. The expression ratio of VKORC1:VKORC1L1 was 5:4 in
424 spleen, 4:5 in pancreas, 2:3 in liver, 1:2 in heart, ovary and lung, 1:4 in brain and muscle,
425 1:9 in kidney, and 1:19 in testis (Fig. 3B).

DISCUSSION

1. Avian interspecific differences in VKOR activity

1-1. Comparison of VKOR activity among avian species

This study suggested hepatic VKOR activities of raptors were lower than that of turkeys and chickens. VKOR plays an important role in the vitamin K cycle for activations of vitamin K-dependent coagulation factors. Inhibiting VKOR by ARs induces lethal hemorrhages in raptors (Murray, M, 2018; Rattner et al., 2012; Rattner et al., 2014; Rattner et al., 2015). Because the present study focused on comparing the maximum VKOR activity between avian species, 12.5, 100, and 400 μM of VKO were used as substrates for 1.0 mg/mL liver microsomes. The estimated concentration of VKO is 12.5, 100, and 400 μmol/g liver microsomes, respectively. However, the physiological concentration of VKO is approximately 13 pmol/g in chicken liver (Will et al., 1992) and 0.1 pmol/g in mice liver (Okano et al., 2008). The concentration of VKO used for the VKOR activity assay in the present study is much higher than physiological concentrations. It is also important to investigate the activity under physiological concentrations of the substrate.

It is reported that concentration of vitamin K in liver of chickens is less than that of rats, while the concentration of VKO in liver is higher in chickens than in rats (Will et al., 1992). This indicates that chickens have a poor ability to reduce VKO in the vitamin K cycle, so they require high doses of vitamin K in their diets. Similarly, it is possible that raptors require high doses of dietary vitamin K. K vitamins are divided into two major types. Vitamin K_1 (phylloquinone) is synthesized in plants and is the primary dietary source of vitamin K. Vitamin K_2 (menaquinones, MK) represents a family of many different subtypes. One subtype, MK4, is converted from phylloquinone and is the major physiological vitamin K form (Van Horn 2013). Although many other menaquinones are converted by gut bacteria, the menaquinones of gut origin make a relatively minor contribution to the hepatic stores of vitamin K. Phylloquinone and MK4 are also available from meat, including rats and mice, which are diets of raptors (Will et

al., 1992; Elder et al., 2006; Okano et al., 2008). Considering these previous studies, raptors are presumed to gain vitamin K from rodents and other prey in the wild or menaquinones synthesized in gut to respond to the dose of required vitamin K.

1-2. VKOR activity related to amino acid sequence and mRNA expression of VKORC1 and VKORC1L1

This study suggested that not only VKORC1, but also VKORC1L1 is important for VKOR activity in avian species. It was reported that the V_{max} of VKORC1 was 16-fold higher than that of VKORC1L1 in rats (Hammed et al., 2013). Moreover, mRNA expression of VKORC1 was over 10-fold higher than that of VKORC1L1 in rat and mouse livers (Hammed et al., 2013; Caspers et al., 2015). Therefore, VKORC1 may support 96% of VKOR activity in mammals. Conversely, the individual V_{max} of VKORC1 and VKORC1L1 have been unknown in avian species. Because the avian CXXC motif was different between VKORC1 (CLVC, Fig. S4-A) and VKORC1L1 (CIVC, Fig. S4-B), it is possible that the V_{max} values could be different between avian VKORC1 and VKORC1L1. While mRNA of VKORC1 was expressed higher than that of VKORC1L1 in turkey liver (Fig. 2), both mRNA of VKORC1 and VKORC1L1 were equivalently expressed in chicken liver (Fig. 3). Therefore, in contrast to mammals, it is possible that avian VKOR activity is supported by both VKORC1 and VKORC1L1, and the mRNA expression ratio could cause avian interspecific differences in VKOR activity.

Although it is difficult to compare the absolute amount of VKORC1 and VKORC1L1 between turkeys and chickens, the total expression amount of VKORC1 and VKORC1L1 was 2.5-fold higher in turkey liver (total amount was 3050, VKORC1 was 2700, and VKORC1L1 was 350 copy number/ng total RNA) than in chicken liver (total amount was 1200, VKORC1 was 500, and VKORC1L1 was 700 copy number/ng total RNA). The total expression amount of VKORC1 and VKORC1L1 could also cause avian interspecific differences in VKOR activity. Information on the mRNA expression ratio and the total quantities of VKORC1 and VKORC1L1 from various avian species might

provide some basis to account for interspecific differences in VKOR activity.

2. Avian interspecific differences in sensitivity of VKOR activity to warfarin

2-1. Comparison of VKOR activity inhibited by warfarin among avian species

This study suggested that the percentage of hepatic microsomal VKOR activity remaining after warfarin incubation had a rank order of chickens > turkeys, *Accipiter* (goshawk and sparrowhawks) and peregrine falcon > *Bubo* (snowy owls and great horned owls) > canaries. This result indicates that the VKOR activity of raptors, especially *Bubo*, is inhibited by warfarin more easily than that of chickens, which is supported in part by the observation of 20-30-fold greater toxicity of the AR diphacinone in American kestrel compared to that described for Northern bobwhite and mallards (Rattner et al., 2018a). Therefore, it is not recommended to extrapolate poultry data to raptors for risks assessment of ARs in raptors. Moreover, because there were interspecific differences between raptors and canaries, it is also important to examine individual species data about the inhibited rate of VKOR activity for accurate risk assessment of ARs in non-target avian species.

2-2. Amino acid sequence of VKORC1

For rats, the mutation of tyrosine at amino acid 139 in the sequence of VKORC1 shows resistance to warfarin, and it has been inferred that the Thr-Tyr-Ala (TYA) motif (at amino acid 138 to 140) is the warfarin-binding site (Rost et al., 2005). Hence, the warfarin-binding site of VKORC1 sequence was compared among avian species. The motifs in chickens, turkeys, and canaries were TYV, TYA, TYG, respectively (Fig. S4-A). The VKOR activity of chickens was more resistant to warfarin compared to that of turkeys (Fig. 1B). It was reported that chickens had a 40-fold higher K_i than rats for VKOR activity in response to warfarin (Watanabe et al., 2010). Combined with the fact that the warfarin-binding site of VKORC1 is TYV in chickens and TYA in turkeys and rats (Fig. S4-A), warfarin resistance might be higher with TYV than TYA.

510 Surprisingly, the VKOR activity of canaries was strongly inhibited compared
511 to other avian species, including raptors. In canaries, the warfarin-binding site of
512 VKORC1 is TYG as well as ostriches (Fig. S4-A). A previous study reported that the
513 VKOR activity of ostriches was also strongly inhibited: inhibited VKOR activity was
514 29% at 100 μM of VKO with 1 μM of warfarin (Watanabe et al., 2010). These data suggest
515 that TYG is a significant determinant of sensitivity to warfarin and possibly other ARs.
516 The amino acid at position 140 of VKORC1 could be important for avian interspecific
517 difference in VKOR inhibition. Information about VKORC1 sequences and structures of
518 other avian species, especially raptors, are needed for more accurate avian VKOR
519 characterization and risk assessments of ARs.
520
521 *2-3. Amino acid sequence of VKORC1L1*
522 Because the sequence of VKORC1L1 in all the avian species shown in Fig. S4-
523 B had the same warfarin-binding site (TYL), the warfarin-binding site of VKORC1L1
524 does not cause avian interspecific differences in VKOR susceptibility. There is little
525 information about amino acid mutations in VKORC1L1. It was reported that warfarin
526 resistance was caused by a V36L mutation in VKORC1L1 obtained from HEK-293-
527 EBNA cell microsomes expressing recombinant human VKORC1L1 (Hünerberg 2009).
528 As shown in Fig. S4-C, the 36th amino acid was valine in the VKORC1L1 of human and
529 rats, and in VKORC1 of human, rats, and avian species. However, interestingly the 36th
530 amino acid was leucine in avian VKORC1L1. Therefore, it is possible that the leucine at
531 the 36th amino acid position in avian VKORC1L1 causes warfarin resistance in VKOR
532 activity. More information about avian VKORC1L1 is needed, including mutations.
533
534 *2-4. mRNA expression of VKORC1 and VKORC1L1*
535 In avian species, the K_i for VKOR activity in response to warfarin is unknown
536 for both VKORC1 and VKORC1L1. It was reported that the K_i for VKOR activity in
537 response to warfarin was 50-fold higher in VKORC1L1 than in VKORC1 obtained from

538 yeast microsomes expressing recombinant proteins from rats (Hammed et al., 2013).
539 Moreover, VKOR activity in the testis (predominantly VKORC1L1 mRNA) was more
540 resistant to warfarin compared to that of the liver (predominantly VKORC1 mRNA).
541 Therefore, high expression of VKORC1L1 may induce warfarin resistance in mammals.
542 The VKOR activity of chickens was more resistant to warfarin than that of turkeys (Fig.
543 1B). The mRNA expression ratio of VKORC1:VKORC1L1 differed between turkey (8:1,
544 Fig. 2B) and chicken liver (2:3, Fig. 3B). This result suggests that expression ratio of
545 VKORC1:VKORC1L1 could account for interspecific differences in VKOR inhibition
546 by ARs among various species of birds. Both the K_i of avian individual specific VKORC1
547 and VKORC1L1 and expression ratio should be considered for the risk assessment of
548 ARs in wild avian species.
549 Although our study focused on avian interspecific differences in VKOR, which
550 is the target molecule of ARs, the warfarin-binding capacity of albumin in serum is also
551 an important factor for avian interspecific differences in susceptibility to ARs. Chicken
552 albumin may have a greater warfarin-binding capacity (9-fold greater than human),
553 resulting in a longer half-life and less toxicity despite high metabolic ability (Rajaian et
554 al., 1997; Watanabe et al., 2015). Although there is no information on raptor albumin,
555 albumin in raptors and chickens may be less identical than in other avian species such as
556 turkey, ostrich, and zebra finch (91%-70% identical amino acid sequences). Lower
557 warfarin binding capacity of albumin in some species of birds (perhaps raptors) might
558 contribute to differences in AR sensitivity.
559
560 **3. mRNA distribution of VKORC1 and VKORC1L1 in turkey and chicken tissues**
561 Interestingly, the mRNA distribution in turkey tissues was similar to that in rat
562 and mouse tissues: VKORC1 was expressed higher than VKORC1L1 in liver, kidney and
563 lung; and VKORC1L1 was expressed higher than VKORC1 in testis and brain (Fig. 2)
564 (Caspers et al., 2015; Hammed et al., 2013). In contrast, VKORC1L1 was expressed
565 higher than VKORC1 in most chicken tissues, except for spleen (Fig. 3). These results

suggest that interspecific difference in mRNA distribution of VKORC1 and VKORC1L1 may be greater in birds than mammals, and VKORC1L1 can support VKOR activity in liver as well as in extra hepatic tissues in some avian species. In mammals, the focus has principally been on VKORC1. In birds, however, both VKORC1 and VKORC1L1 should be considered.

CONCLUSIONS

In conclusion, our study demonstrated that VKOR activity of raptors is both lower and more readily inhibited by the prototypic AR warfarin compared to poultry. Moreover, both the Ki of VKORC1 and VKORC1L1, and the mRNA expression ratio, may contribute to interspecific difference in AR inhibition of VKOR in birds. The value of K_i for VKOR to ARs could depend on the amino acid at position 140 in the TYX warfarin-binding motif of VKORC1. Further, the value of K_i for VKOR to ARs could depend on other amino acid mutations in VKORC1L1. Therefore, further information about raptor VKORC1 and VKORC1L1, including K_i, mRNA expression ratio and amino acid sequences, are needed to better assess the risk of ARs to raptors. Such data may help elucidate the molecular and genetic factors that contribute to the seemingly greater sensitivity of raptorial and scavenging birds to AR intoxication.

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602 Table 1. Avian species used in this study.

603

Common name	Scientific name	Sex	Sample size	Source
Chicken (Rhode island red)	*Gallus gallus domesticus*	Male	3	a
		Female	3	a
Turkey (Bronze)	*Meleagris gallopavo*	Male	3	b
Canary (Lemon)	*Serinus canaria*	Male	6	c
Peregrine falcon	*Falco peregrinus pealei*	Female	1	d
Sparrowhawk	*Accipiter nisus nisosimilis*	Female	2	d, e
Goshawk	*Accipiter gentilis fujiyamae*	Female	1	d
Steller's sea eagle	*Haliaeetus pelagicus*	Male	1	d
White-tailed eagle	*Haliaeetus albicilla*	Female	1	d
Snowy owl	*Bubo scandiacus*	Male	2	e
Great horned owl	*Bubo virginianus*	Male	1	e
		Female	1	e
Night heron	*Nycticorax nycticorax*	Female	1	e

604 a: Farm at Hokkaido University (Sapporo, Japan)

605 b: Sankyo Labo Service Corporation, Inc. (Tokyo, Japan)

606 c: Local pet shop (Sapporo, Japan)

607 d: Institute for Raptor Biomedicine Japan (Kushiro Shitsugen Wildlife Center, Kushiro,

608 Japan)

609 e: Sapporo Maruyama Zoo (Hokkaido, Japan)

610

611 Table 2. VKOR activity (produced vitamin K, pmol/min/mg protein) at 12.5, 100 and 400 μM of VKO in turkeys, chickens, canaries and
612 5 species of raptors. The upper row in each VKO concentration shows individual data, and the bottom row shows the mean ± S.D.
613

VKO [μM]	Turkeys	Male chickens	Female chickens	Canaries	Snowy owls[a]	Great horned owls[a]	Goshawk[b]	Sparrow-hawks[b]	Peregrine falcon
12.5				32, 45	51, 51	20, 31	26	23, 28	
	115 ± 22	78 ± 28	49 ± 31	38	38 ± 15		26 ± 3		9
100				79, 72	89, 70	44, 79	38	33, 38	
	222 ± 36	124 ± 50	97 ± 66	75	70 ± 19		36 ± 3		14
400				77, 94	100, 94	52, 84	42	42, 51	
	270 ± 17	153 ± 57	103 ± 65	85	83 ± 21		45 ± 5		14

614 No significant differences in VKOR activity at each substrate (VKO) concentration among turkeys, male chickens, female chickens, *Bubo*[a]
615 (snowy owls and great horned owls) and *Accipiter*[b] (a goshawk and sparrowhawks) were observed (Steel-Dwass test, $P < 0.05$).
616 VKOR activities in *Haliaeetus* (Steller's sea eagle and white-tailed eagle) were undetectable.

Table S1. Primers for avian VKORC1L1 cloning.

Primers were designed based on the VKORC1L1 nucleotide sequences.

Gene		Sequence (5'→3') *	Amplicon size (bp)
Avian VKORC1L1	Forward	CTTGGTATGACAGCAAGTGC	225
Avian VKORC1L1	Reverse	CTGTTTGGGTTGGRAGTTGC	

* The letters R encodes A and G.

620 Table S2. Accession numbers of VKORC1 and VKORC1L1 genes.

621

Common name	**Scientific name**	**Accession number**	
		VKORC1	**VKORC1L1**
Chicken	*Gallus gallus*	**NM_206807**	**NM_001001328**
Ostrich	*Struthio camelus australis*	-	**XM_009675917**
Turkey	*Meleagris gallopavo*	**XM_010726800**	**XM_003211735**
Anna's hummingbird	*Calypte anna*	**XM_008503547**	**XM_008490706**
Yellow-throated sandgrouse	*Pterocles gutturalis*	**XM_010087012**	**XM_010074393**
Brown roatelo	*Mesitornis unicolor*	**XM_010190461**	**XM_010179204**
Crested ibis	*Nipponia nippon*	**XM_009474946**	**XM_009469732**
Night heron	*Nycticorax nycticorax*	-	**LC097088**
Emperor penguin	*Aptenodytes forsteri*	**XM_009285145**	**XM_009287681**
Golden eagle	*Aquila chrysaetos canadensis*	-	**XM_011581635**
Barn owl	*Tyto alba*	-	**XM_009973792**
Snowy owl	*Bubo scandiacus*	-	**LC097089**
Great horned owl	*Bubo virginianus*	-	**LC097090**

Bald eagle	*Haliaeetus leucocephalus*	-	**XM_010573578**
White-tailed eagle	*Haliaeetus albicilla*	-	**XM_009928642**
Peregrine falcon	*Falco peregrinus*	-	**XM_005240548**
Common canary	*Serinus canaria*	**XM_009100016**	**XM_009094710**
Human	*Homo sapiens*	**NM_024006**	**NM_173517**
Norway rat	*Rattus norvegicus*	**NM_203335**	**NM_203338**
House mouse	*Mus musculus*	**NM_178600**	**NM_027121**
Western painted turtle	*Chrysemys picta bellii*	**XM_005279372**	**XM_005283424**
Western clawed frog	*Xenopus tropicalis*	**NM_001006927**	**NM_001016769**
Fugu rubripes	*Takifugu rubripes*	**NM_001032666**	**NM_001032768**
Japanese medaka	*Oryzias latipes*	**XM_004080314**	**XM_004076602**
Cephalochordata	*Branchiostoma floridae*	**XM_002611843, XM_002587531***	
Purple sea urchin	*Strongylocentrotus purpuratus*	**XM_001181369** *	

* Cephalochordata and Purple sea urchin possesses ancestral type of VKOR gene.

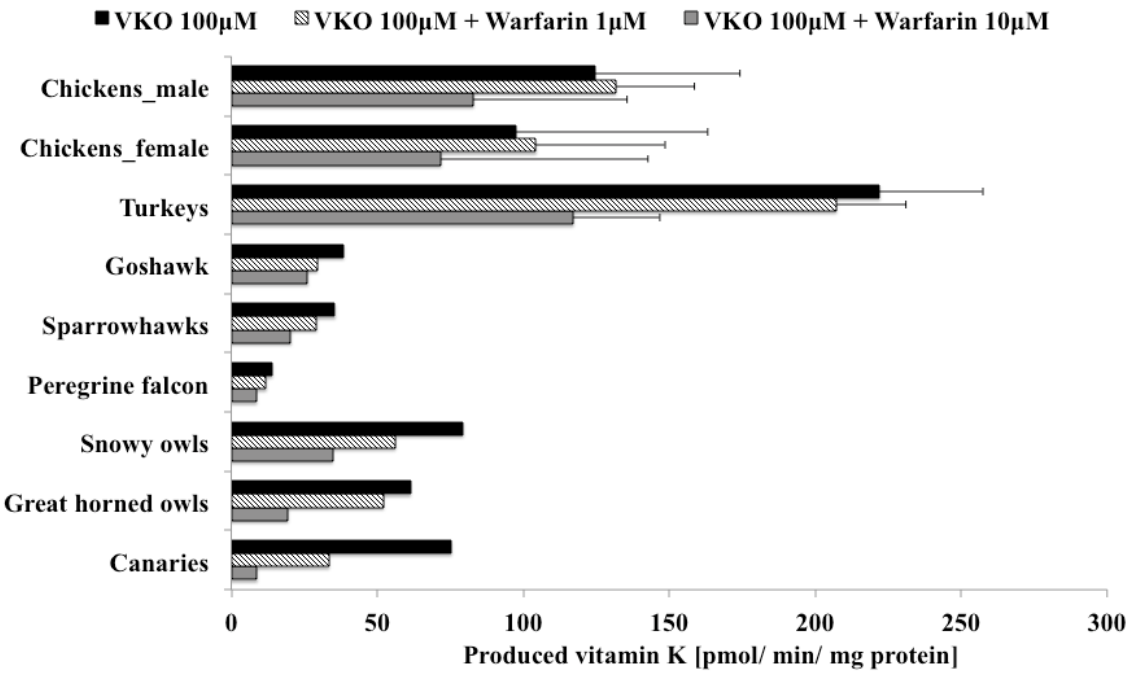
623 Table S3. Primers for cloning and qPCR in turkeys and chickens.

624

Gene		Sequence (5'→3')	Amplicon size (bp)	Efficiency (%)	Accession number	Reference
Chicken_VKORC1	Forward	TTTGTGGGTCGGGACAGCGCCA	218	95.6	**NM_206807**	This study
	Reverse	ACGACGTAGGTGCTGAGGCAGA				
Chicken_VKORC1	Forward	GTCGGGACAGCGCCATCAACGT	215	Not tested	**NM_206807**	This study
_ for cloning	Reverse	GTTGACGTAGGTGCTGAG				
Chicken_	Forward	AGAAGGGCCGCGATCTCCACTACCA	106	89.2	**NM_001001328**	This study
VKORC1L1	Reverse	CCCAACAGACCGAATCCTCGACCCCAT				
Chicken_GAPDH	Forward	CTCTGTTGTTGACCTGACCT	125	98.8	**NM_204305**	Watanabe
	Reverse	CAACCTGGTCCTCTGTGTAT				et al., 2013
Chicken_β-actin	Forward	GAGAAATTGTGCGTGACATCA	152	95.3	**NM_205518**	This study
	Reverse	CCTGAACCTCTCATTGCCA				
Turkey_VKORC1	Forward	TTTGTGGGTCGGGACAGCGCCA	218	107.2	**XM_010726800**	This study
	Reverse	ACGGCGTAGGTGCTGAGGCAGA				
Turkey_VKORC1_	Forward	GTTTGTGGGTCGGGACAGCGCCA	219	Not tested	**XM_010726800**	This study
for cloning	Reverse	ACGGCGTAGGTGCTGAGGCAGA				

Turkey_ VKORC1L1	Forward	GAAGGGGCCGCGATCTCCACTACCA	106	104.0	**XM_003211735**	This study
	Reverse	CCCAACAGCCCGAATCCTCGACCCCAT				
Turkey_GAPDH	Forward	CTCTGTTGTTGACCTGACCT	125	98.0	**NM_001303179**	This study
	Reverse	CAACCTGGTCCTCTGTGTAT				
Turkey_β-actin	Forward	GAGAAATTGTGCGTGACATCA	152	104.0	**NM_001303173**	This study
	Reverse	CCTGAACCTCTCATTGCCA				

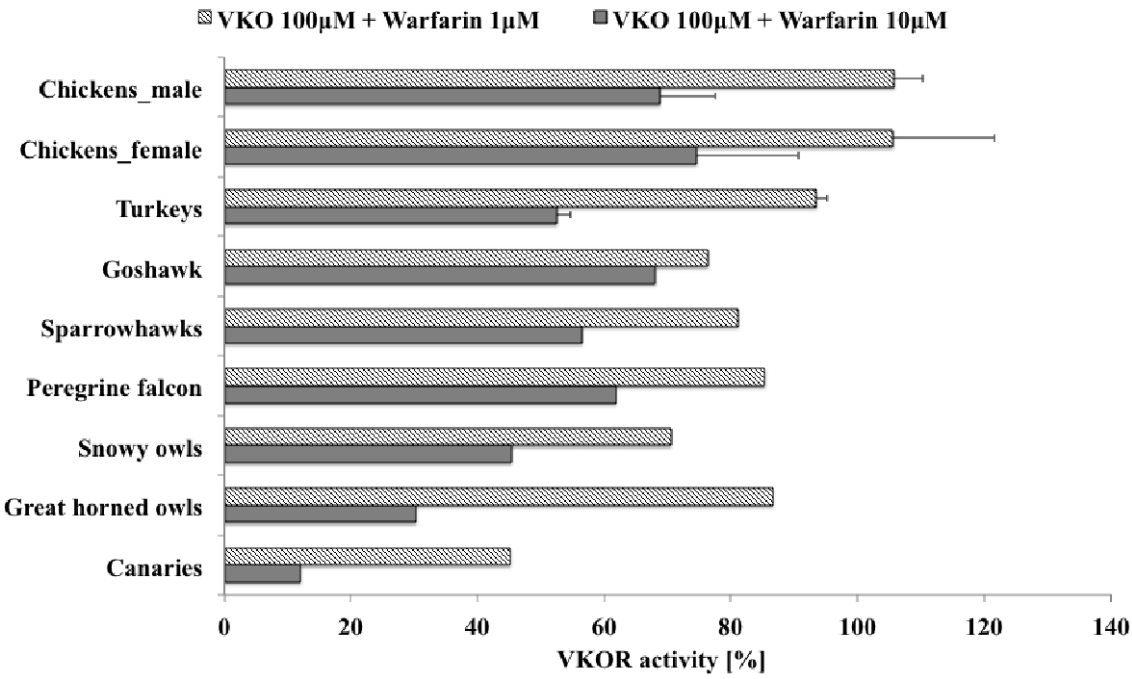
637



638

639

640 B



641

642

643 Fig. 1. VKOR activity inhibited by 1 and 10 µM warfarin at 100 µM of VKO (A) and

644 percentage of inhibited activity (B) in turkeys, chickens, canaries and five species of

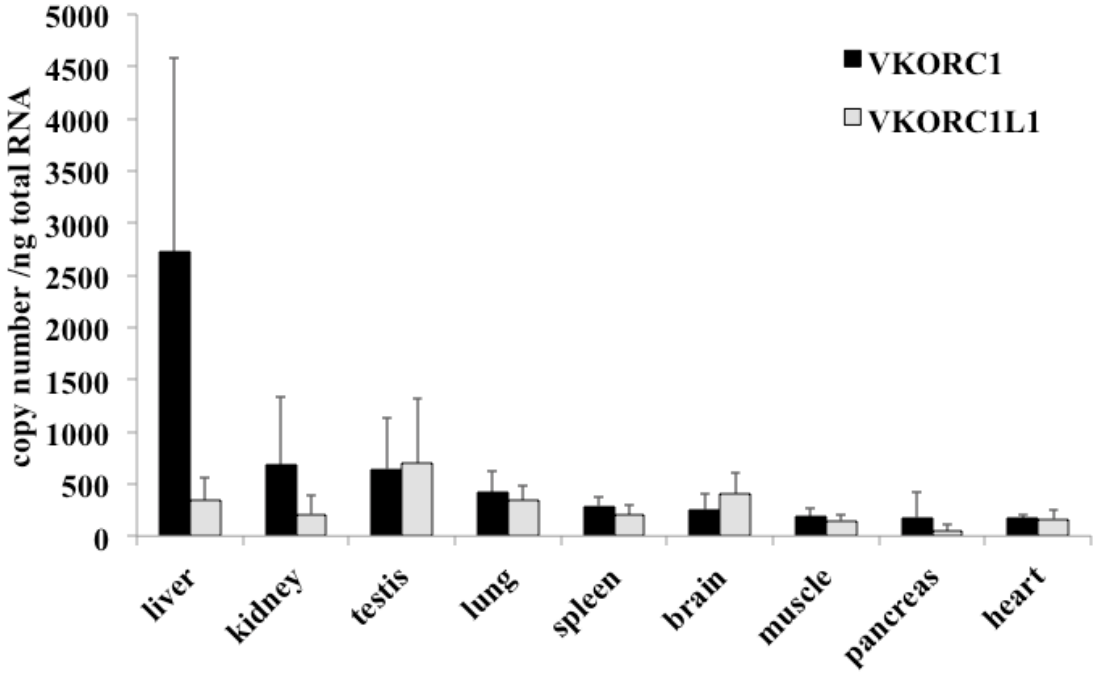
raptors.

The percentage (%) was calculated using the following formula;

VKOR activity (%) = VKOR activity with warfarin/ that without warfarin *100

Each data point from turkeys and chickens represents the mean of three animals ± S.D. (error bars). The numbers of other species were less than three. Therefore there are no S.D. values. No significant differences in percentage of VKOR activity at each warfarin concentration among chickens, *Accipiter*[a] (a goshawk and sparrowhawks), turkeys and *Bubo*[b] (snowy owls and great horned owls) were observed (Steel-Dwass test, $P < 0.05$).

653 A



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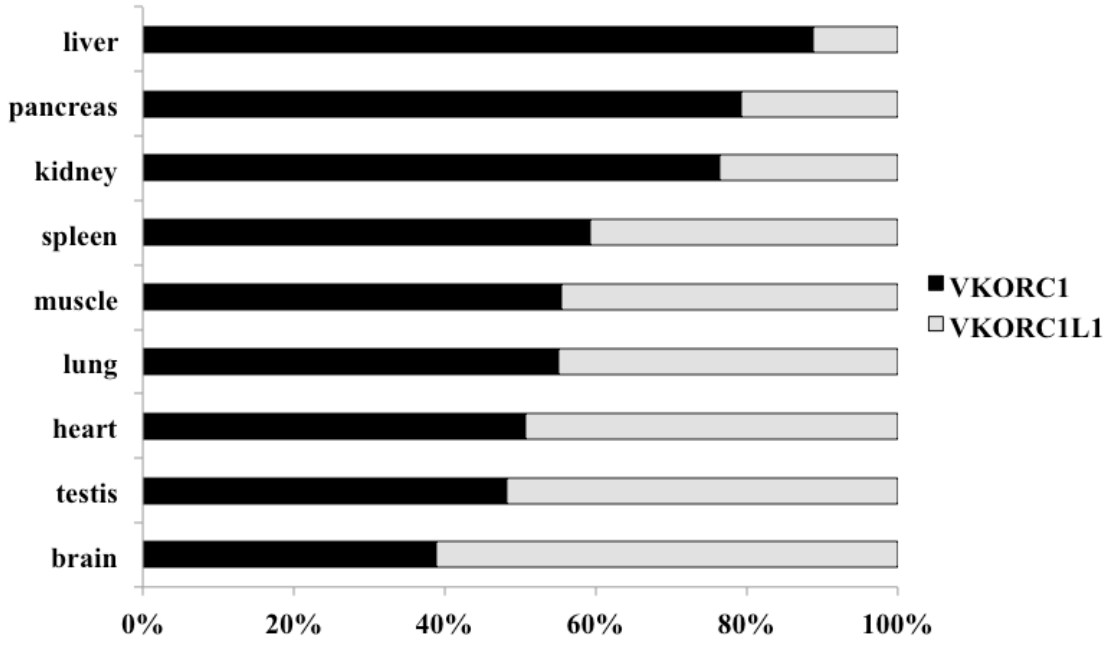
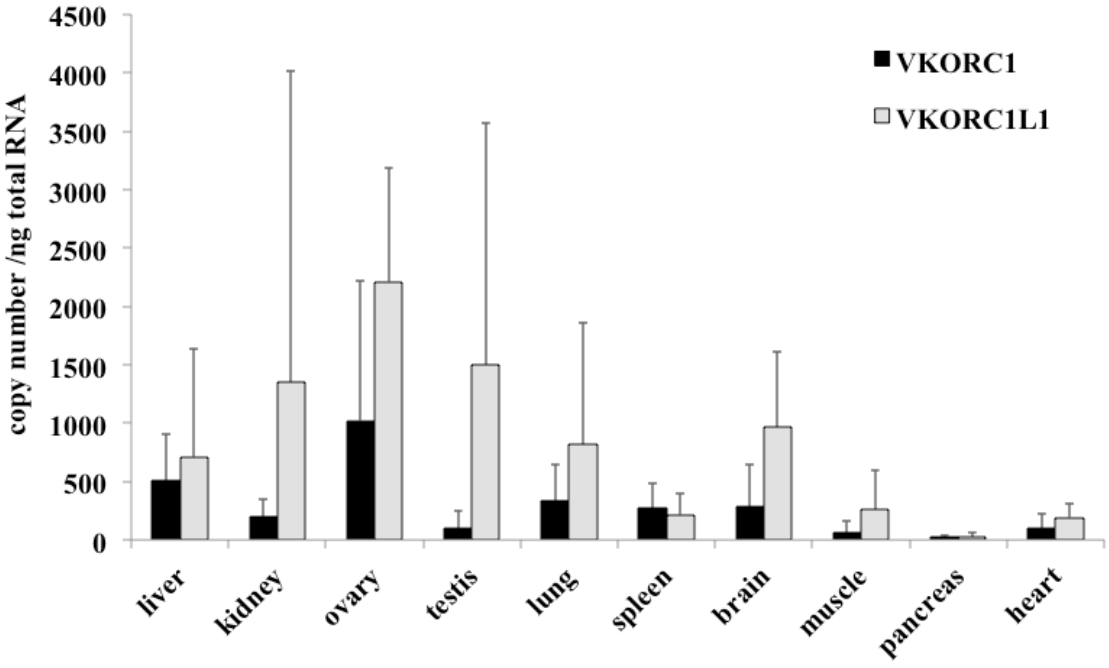


656

657 Fig. 2. mRNA expression of VKORC1 and VKORC1L1 (A) and their ratios (B) in nine
658 different tissues of male turkeys. Each data represents the mean of three animals ± S.D.
659 (error bars).

660 A



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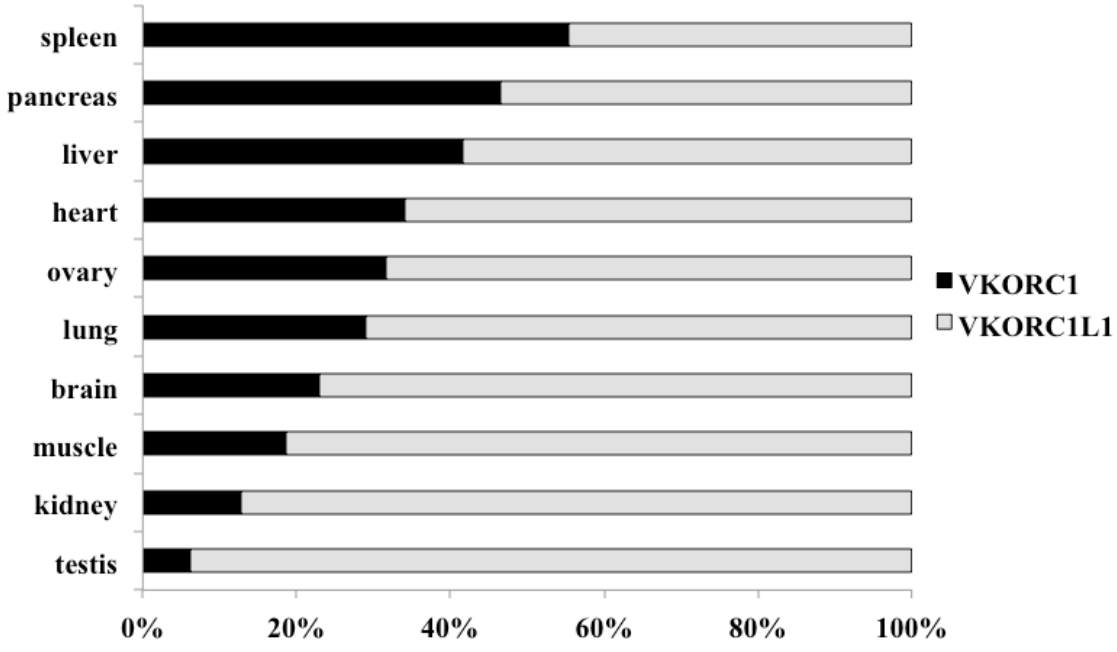
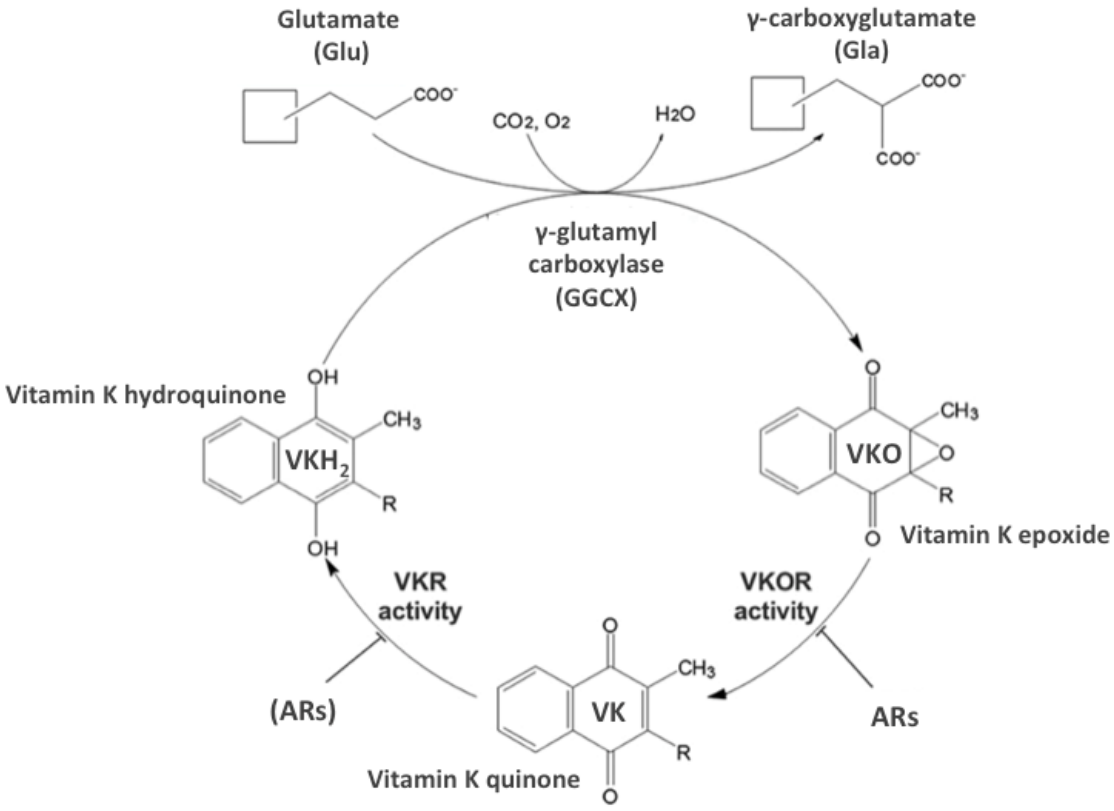
662 B



663

664 Fig. 3. mRNA expression of VKORC1 and VKORC1L1 (A) and their ratios (B) in ten
665 different tissues of three male and female chickens. Each data point represents the mean
666 of six animals ± S.D. (error bars) except for ovary and testis. Data of ovary and testis
667 represents the mean of three animals ± S.D. (error bars).

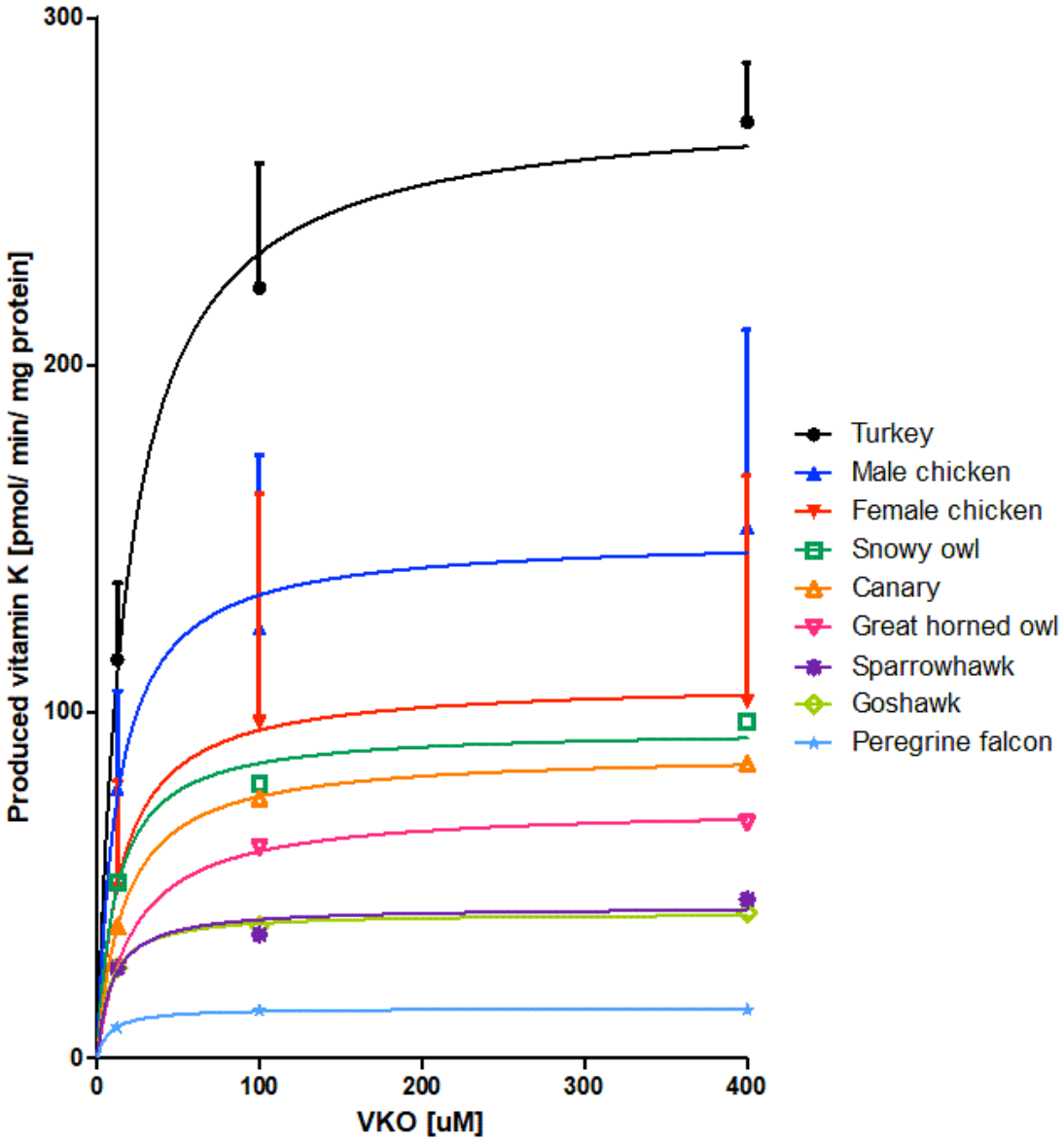


668

669 Fig. S1. Modified vitamin K cycle (Hammed et al., 2013; Tie et al., 2013).

670 During vitamin K dependent carboxylation, vitamin K hydroquinone is oxidized to
671 vitamin K 2, 3-epoxide (VKO) by γ-glutamyl carboxylase. VKO is reduced to vitamin K
672 by VKOR. This reaction is inhibited by ARs (e.g. warfarin).

673



674

675 Fig. S2. VKOR activity: vitamin K produced versus 12.5, 100 and 400 μM of VKO in
676 turkeys, chickens, canaries and five species of raptors.

677 VKOR activities of *Haliaeetus* (Steller's sea eagle and white-tailed eagle) were
678 undetectable. Each data point of turkeys and chickens represents the mean of three
679 animals ± S.D. (error bars). The numbers of other species were less than three, therefore
680 there are no S.D. values.

681

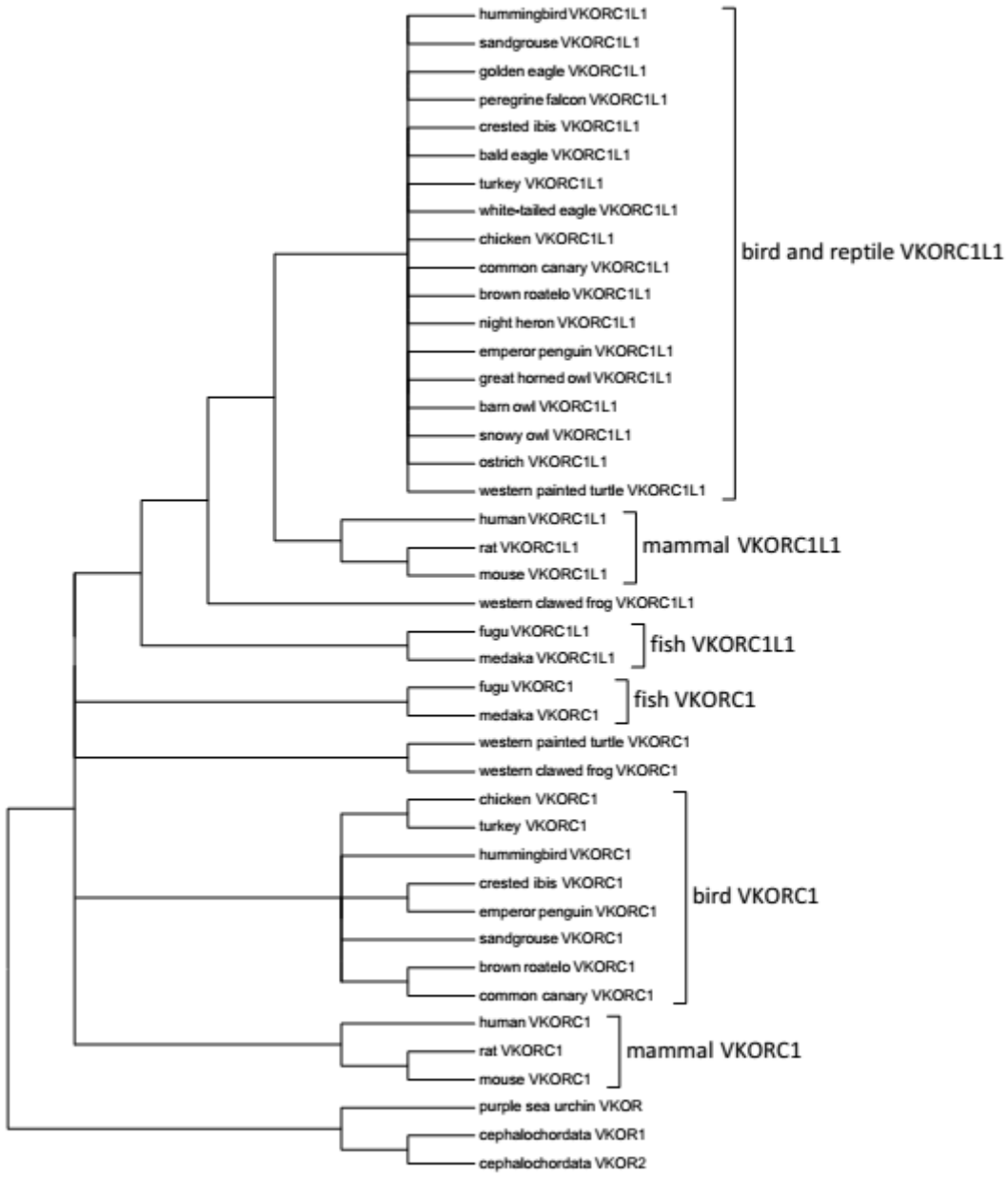
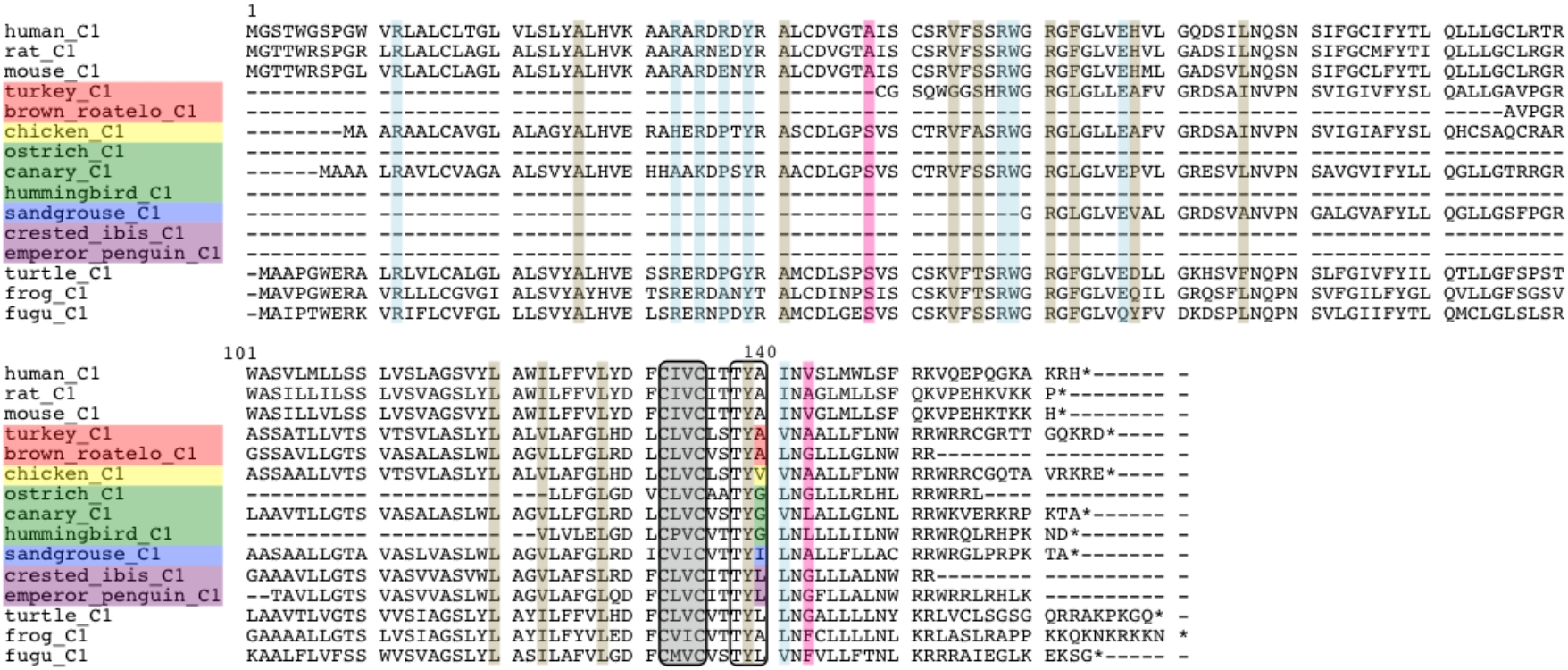


Fig. S3. Phylogenetic tree of the VKOR protein family. In all vertebrates, VKORC1L1 made one cluster. VKORC1 were collapsed because of low bootstrap value (less than 60%). In VKORC1, 4 clusters; fish, bird, mammal, and other animal VKOC1, were made.



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687 Fig. S4-A. Amino acid sequence alignment of VKORC1 of different species.

688 Grey box indicates the catalytic CXXC motif, which is supposed to be the active site of the VKORC1 protein. White box indicates the

689 warfarin-binding site. In avian species, the amino acid at position 140 is highlighted in five different colors (red, alanine; yellow, valine;

690 green, glycine; blue, isoleucine; purple, leucine). Shading highlights amino acids whose mutations was reported in rats, mice or humans

691 (brown, warfarin resistance; sky blue, low VKOR activity; pink, high VKOR activity) (Lasseur et al., 2006a; Rost et al., 2009; Hodroge

et al., 2012; Tanaka et al., 2012; Müller et al., 2014).

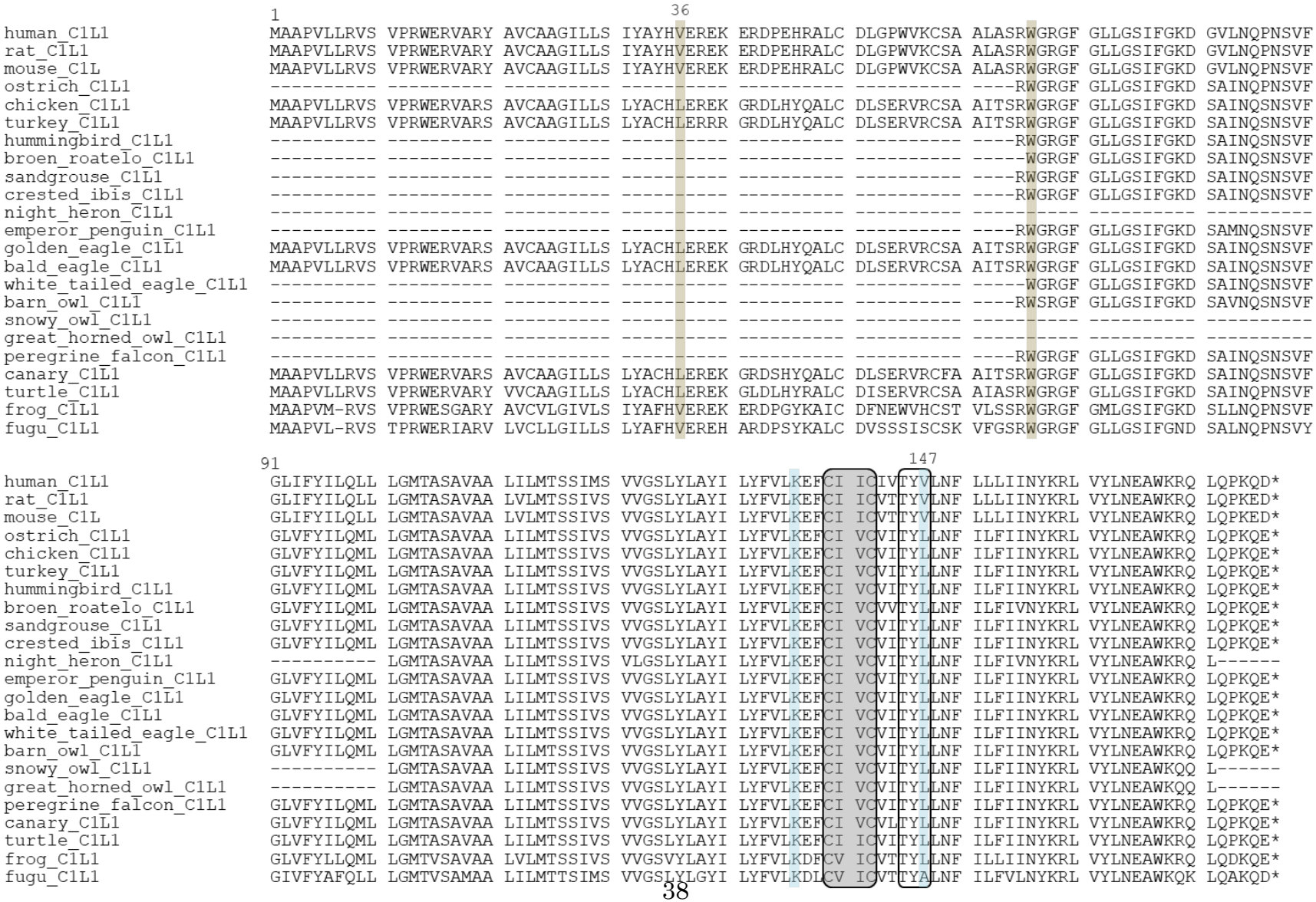
1
36
human_C1L1 MAAPVLLRVS VPRWERVARY AVCAAGILLS IYAYHVEREK ERDPEHRALC DLGPWVKCSA ALASRWGRGF GLLGSIFGKD GVLNQPNSVF
rat_C1L1 MAAPVLLRVS VPRWERVARY AVCAAGILLS IYAYHVEREK ERDPEHRALC DLGPWVKCSA ALASRWGRGF GLLGSIFGKD GVLNQPNSVF
mouse_C1L MAAPVLLRVS VPRWERVARY AVCAAGILLS IYAYHVEREK ERDPEHRALC DLGPWVKCSA ALASRWGRGF GLLGSIFGKD GVLNQPNSVF
ostrich_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ----RWGRGF GLLGSIFGKD SAINQPNSVF
chicken_C1L1 MAAPVLLRVS VPRWERVARS AVCAAGILLS LYACHLEREK GRDLHYQALC DLSERVRCSA AITSRWGRGF GLLGSIFGKD SAINQSNSVF
turkey_C1L1 MAAPVLLRVS VPRWERVARS AVCAAGILLS LYACHLERRR GRDLHYQALC DLSERVRCSA AITSRWGRGF GLLGSIFGKD SAINQSNSVF
hummingbird_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ----RWGRGF GLLGSIFGKD SAINQSNSVF
broen_roatelo_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- -----WGRGF GLLGSIFGKD SAINQSNSVF
sandgrouse_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ----RWGRGF GLLGSIFGKD SAINQSNSVF
crested_ibis_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ----RWGRGF GLLGSIFGKD SAINQSNSVF
night_heron_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
emperor_penguin_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ----RWGRGF GLLGSIFGKD SAMNQSNSVF
golden_eagle_C1L1 MAAPVLLRVS VPRWERVARS AVCAAGILLS LYACHLEREK GRDLHYQALC DLSERVRCSA AITSRWGRGF GLLGSIFGKD SAINQSNSVF
bald_eagle_C1L1 MAAPVLLRVS VPRWERVARS AVCAAGILLS LYACHLEREK GRDLHYQALC DLSERVRCSA AITSRWGRGF GLLGSIFGKD SAINQSNSVF
white_tailed_eagle_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- -----WGRGF GLLGSIFGKD SAINQSNSVF
barn_owl_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ----RWSRGF GLLGSIFGKD SAVNQSNSVF
snowy_owl_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
great_horned_owl_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
peregrine_falcon_C1L1 ---------- ---------- ---------- ---------- ---------- ---------- ----RWGRGF GLLGSIFGKD SAINQSNSVF
canary_C1L1 MAAPVLLRVS VPRWERVARS AVCAAGILLS LYACHLEREK GRDSHYQALC DLSERVRCFA AITSRWGRGF GLLGSIFGKD SAINQSNSVF
turtle_C1L1 MAAPVLLRVS VPRWERVARY VVCAAGILLS LYACHLEREK GLDLHYRALC DISERVRCSA AIASRWGRGF GLLGSIFGKD SAINQPNSVF
frog_C1L1 MAAPVM-RVS VPRWESGARY AVCVLGIVLS IYAFHVEREK ERDPGYKAIC DFNEWVHCST VLSSRWGRGF GMLGSIFGKD SLLNQPNSVF
fugu_C1L1 MAAPVL-RVS TPRWERIARV LVCLLGILLS LYAFHVEREH ARDPSYKALC DVSSSISCSK VFGSRWGRGF GLLGSIFGND SALNQPNSVY
91
147
human_C1L1 GLIFYILQLL LGMTASAVAA LILMTSSIMS VVGSLYLAYI LYFVLKEFCI ICIVTYVLNF LLLIINYKRL VYLNEAWKRQ LQPKQD*
rat_C1L1 GLIFYILQLL LGMTASAVAA LVLMTSSIVS VVGSLYLAYI LYFVLKEFCI ICVTTYVLNF LLLIINYKRL VYLNEAWKRQ LQPKED*
mouse_C1L GLIFYILQLL LGMTASAVAA LVLMTSSIVS VVGSLYLAYI LYFVLKEFCI ICVTTYVLNF LLLIINYKRL VYLNEAWKRQ LQPKED*
ostrich_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
chicken_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
turkey_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
hummingbird_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
broen_roatelo_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVVTYLLNF ILFIVNYKRL VYLNEAWKRQ LQPKQE*
sandgrouse_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
crested_ibis_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
night_heron_C1L1 ---------- LGMTASAVAA LILMTSSIVS VLGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIVNYKRL VYLNEAWKRQ L------
emperor_penguin_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
golden_eagle_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
bald_eagle_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
white_tailed_eagle_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
barn_owl_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
snowy_owl_C1L1 ---------- LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKQQ L------
great_horned_owl_C1L1 ---------- LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKQQ L------
peregrine_falcon_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
canary_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI VCVLTYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
turtle_C1L1 GLVFYILQML LGMTASAVAA LILMTSSIVS VVGSLYLAYI LYFVLKEFCI ICVITYLLNF ILFIINYKRL VYLNEAWKRQ LQPKQE*
frog_C1L1 GLVFYLLQML LGMTVSAVAA LVLMTSSIVS VVGSVYLAYI LYFVLKDFCV ICVTTYLLNF ILLIINYKRL VYLNEAWKRQ LQDKQE*
fugu_C1L1 GIVFYAFQLL LGMTVSAMAA LILMTTSIMS VVGSLYLGYI LYFVLKDLCV ICVTTYALNF ILFVLNYKRL VYLNEAWKQK LQAKQD*

Fig. S4-B. Amino acid sequence alignment of VKORC1L1 of different species.

Grey box indicates the catalytic CXXC motif, which is supposed to be the active site of the VKORC1L1 protein. White box indicates the warfarin-binding site. Brown or sky blue shading highlights amino acids whose mutations give warfarin resistance or low VKOR activity, respectively (Hünerberg, 2009).

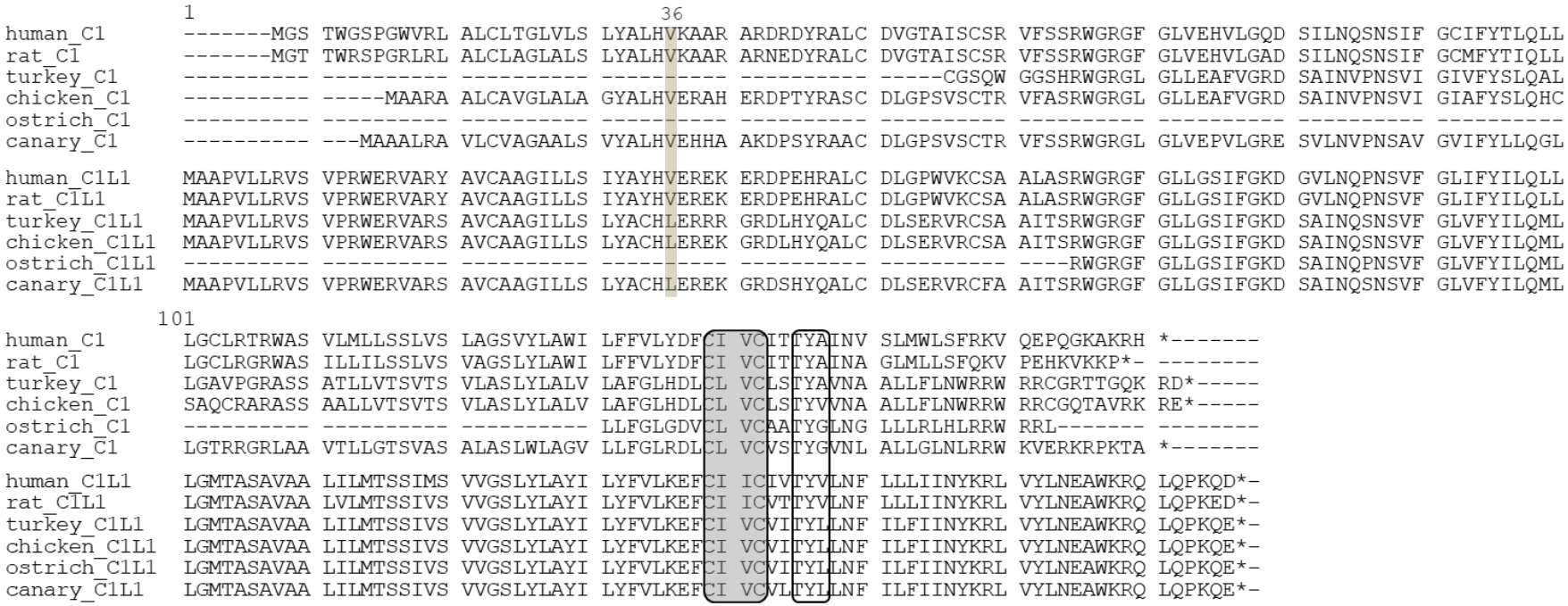

Fig. S4-C. Amino acid sequence alignment of VKORC1 and VKORC1L1 of human, rat, turkey, chicken, ostrich and canary. For the 36th amino acid (brown shading), a V36L mutation was reported to cause warfarin resistance (Hünerberg et al., 2009). Grey box indicates the catalytic CXXC motif, which is supposed to be the active site of the VKORC1 and VKORC1L1 protein. White box indicates the warfarin-binding site.

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