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

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29 **ABSTRACT**

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

49

50 Keywords: anticoagulant rodenticides, VKORC1, VKORC1L1, raptors,

51 **INTRODUCTION**

52 Worldwide use of anticoagulant rodenticides (ARs) for vertebrate pest control
53 has led to the unintentional exposure of non-target animals, especially raptors, to these
54 poisons (López-Perea et al., 2018). Exposure pathways of ARs to raptors have been
55 presumed to be prey on target rodents or non-target animals. Rattner et al. (2018a)
56 summarize the median lethal dose (LD₅₀) for anticoagulant rodenticides (ARs) among
57 animals. Avian species seem more tolerant to ARs than mammals. However, the LD₅₀ for
58 the first-generation AR diphacinone in the American kestrel (*Falco sparverius*) is lower
59 than in poultry (Rattner et al., 2011), and a comparative risk model found that second-
60 generation ARs pose a far greater risk to predatory and scavenging birds (Erickson et al.,
61 2004; Anderson et al., 2011). Considering both the low values of LD₅₀ in raptors and the
62 frequent poisoning of non-target wild birds, especially raptors, implies raptors have
63 different susceptibility to ARs than poultry.

64 Interspecific difference in sensitivity to ARs could be caused by differences in
65 the both AR metabolism by cytochrome P450 (CYP) and the Vitamin K 2, 3-epoxide
66 reductase (VKOR) inhibition, which is the pharmacological target of AR. However, those
67 data are limited to very few avian species. Watanabe et al. (2010) used warfarin as a model
68 compound of ARs and reported that owls in particular showed very low CYP-dependent
69 warfarin metabolic activity compared with other avian species and rats. Rattner et al.
70 (2014) showed that the eliminated half-life of diphacinone in the liver was longer in
71 eastern screech-owls (*Megascops asio*) than in mammals. These studies imply that owls
72 may possess a low ability to detoxify ARs. On the other hand, there are few studies on
73 VKOR characterization in raptors. The VKOR IC₅₀ of several ARs in hepatic
74 microsomes of the American kestrel has been described (Rattner et al., 2018b), i.e.,
75 brodifacoum (0.22 µM), and preliminary warfarin (177 µM) and chlorophacinone (5.1
76 µM). Brodifacoum was reported to have similar inhibition efficiency for VKOR
77 containing hepatic microsomes to mammals (IC₅₀ of 0.15-0.26 µM in mammals).

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

79 quinone (vitamin K) (Fig. S1). This reaction is inhibited by ARs such as warfarin. Vitamin
80 K is reduced to vitamin K hydroquinone (VKH₂) by vitamin K quinone reductase (VKR).
81 VKH₂ is a cofactor for the γ -glutamyl carboxylation of glutamate (Glu) in vitamin K
82 dependent proteins (VKDPs). Some of the most studied VKDPs are clotting factors II,
83 VII, IX, and X in mammals (Ferland, 1998). Although it was thought that avian clotting
84 factors were different from that of mammals (Walz et al., 1975; Frost et al., 1999), recent
85 studies show that avian plasma possesses functional coagulation factors (Thomson et al.,
86 2002), and that avian prothrombin times (chicken, 9.7 s – 27 s) are similar to those of
87 mammals (human, 14 s – 17 s) (Frost et al., 1999; Webster 2009). The inhibiting VKOR
88 by ARs impedes carboxylation of blood clotting factors which can result in hemorrhage
89 in both birds and mammals (Cain et al., 1998; Rattner et al., 2012; Rattner et al., 2014;
90 Rattner et al., 2015).

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

107 VKORC1L1.

108 Although the warfarin-binding site of VKOR has not been clearly identified,
109 amino acids at the 138 to 140 positions in the rat VKORC1 “TYX motif” may be the
110 warfarin-binding site. In rats and mice, tyrosine 139 mutations in VKORC1 exhibit high
111 resistance to warfarin compared to other mutations (Lasseur et al., 2006a; Lasseur et al.,
112 2006b; Rost et al., 2009). The Thr-Tyr-Ala motif also exists in NAD(P)H quinone
113 oxidoreductase (NQOR) that is sensitive to warfarin (Rost et al., 2005). The TYX
114 warfarin-binding site could be important to the K_i for VKOR activity in response to ARs.

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

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

130 **MATERIALS AND METHODS**

131 *Chemicals*

132 HEPES was purchased from Dojindo Laboratories (Kumamoto, Japan).
133 Vitamin K₁ was from Kanto Chemicals (Tokyo, Japan). Diethyl ether and RNAlater®
134 were from Sigma-Aldrich Co. (St. Louis, MO). Racemic warfarin, dithiothreitol (DTT),
135 isopropyl alcohol, hexane, ethanol, methanol, H₂O₂, K₂HPO₄, KH₂PO₄, Na₂CO₃, and
136 NaOH were purchased from Wako Pure Chemical Industries (Osaka, Japan). Isoflurane
137 was purchased from DS Pharma Animal Health Co. (Osaka, Japan).

138

139 *Animals*

140 All avian species including raptors used in this study are shown in Table 1.
141 Chickens (13 months old) were provided from the farm at Hokkaido University (Sapporo,
142 Japan). Turkeys (20 months old) were purchased from Sankyo Labo Service Corporation,
143 Inc. (Tokyo, Japan). Canaries (six months old) were purchased from a local pet shop
144 (Sapporo, Japan). These three species were included in this study because the genome
145 information of both VKORC1 and VKORC1L1 are available. Turkeys and canaries were
146 acclimatized to the environment at a constant temperature (22°C ± 1°C) with a 12:12 h
147 light:dark cycle, and given food and water *ad libitum* for 1 week before commencement
148 of the experiment. Chickens, turkeys and canaries were anesthetized using isoflurane and
149 euthanized by CO₂. After euthanasia, nine different tissues were dissected, including liver,
150 pancreas, spleen, testis or ovary, kidney, lung, heart, brain, and muscle. The excised
151 tissues were cut into small pieces and stored in RNAlater® (Sigma-Aldrich) at -20°C
152 after an overnight incubation at 4°C. The rests of the tissues were immediately frozen in
153 liquid nitrogen and stored at -80°C until use.

154 Livers of raptors were provided by the Institute for Raptor Biomedicine Japan
155 (Kushiro, Japan), and Sapporo Maruyama Zoo (Sapporo, Japan). After the raptors were
156 dead due to illness or traffic accidents, some raptor individuals were dissected
157 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 $9000 \times g$ at 4°C for 20 min. The supernatant was
171 decanted to an ultracentrifugation tube and centrifuged at $105000 \times g$ at 4°C for 60 min.
172 The pellet was homogenized in KPB in ice and then centrifuged again at $105000 \times g$ 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 $1000 \times g$ for 10 min. The supernatant was evaporated under nitrogen

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

194 The concentrations of VKO and vitamin K₁ were determined
195 spectrophotometrically using a molar absorption coefficient of 30,800 M⁻¹ cm⁻¹ at 266 nm
196 and of 18,900 M⁻¹ cm⁻¹ at 249 nm each (Wallin and Martin, 1987).

197

198 *VKOR activity assay*

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

206 The temperatures for pre-incubation and reaction were 41.5°C for turkeys,
207 42°C for chickens, 38.5°C for canaries, 40.5°C for a peregrine falcon, sparrowhawks,
208 goshawk, Steller's sea eagle and white-tailed eagle, and 39.5°C for snowy owls and great
209 horned owls (McNab 1966; Richards 1971; Siegfried et al., 1975; Chaplin et al., 1984;
210 Herrero and Barja, 1998).

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

214 was dissolved in 80 μ L of methanol, and the reaction product was analyzed by HPLC
215 coupled with UV-VIS detector quantified at 270 nm (Pump: LC-20AB, Detector: SPD-
216 20A; SHIMADZU, Kyoto, Japan). Separation was achieved on an Inertsil ODS-3, 2.1 \times
217 150 mm, 5 μ m analytical column (GL Sciences, Kyoto, Japan) run at 40°C at 0.5 mL/min
218 in 99.8% methanol. Vitamin K concentration in all samples except for *Haliaeetus*
219 (Steller's sea eagle and white-tailed eagle) was higher than the limit of quantitation
220 (0.0378 μ M).

221

222 *Inhibition of VKOR activity*

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

226

227 *Total RNA extraction and cDNA synthesis*

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

236

237 *Partial cloning of VKORC1L1*

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

242 94°C, 30 s at 56.8°C, and 30 s at 72°C, with one cycle of final extension for 1 min at
243 72°C.

244 Plasmids were constructed with the PCR products and pCR2.1-TOPO vector
245 using a TOPO TA Cloning Kit (Invitrogen, CA) and transformed into DH5 α -competent
246 cells (TOYOBO, Osaka, Japan). Plasmids were purified using a plasmid miniprep spin
247 kit (Qiagen, Tokyo, Japan). Inserts were sequenced using a BigDye Terminator version
248 1.1 (Applied Biosystems, Foster City, CA). Ethanol precipitation was performed after the
249 amplification reaction, and the plasmid sequence was analyzed by an automated DNA
250 sequencer, ABI Prism 310 Genetic Analyzer (Thermo Fisher Scientific Inc., Kanagawa,
251 Japan), following the manufacturer's instructions.

252

253 *Amino acid sequence alignment of VKORC1 and VKORC1L1*

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

258

259 *Phylogenetic analysis*

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

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

272

273 *Plasmid constructions for quantitative real-time PCR*

274 The cDNA of chickens and turkeys was amplified by PCR using specific
275 primers for VKORC1, VKORC1L1, glyceraldehyde-3-phosphate dehydrogenase
276 (GAPDH), and actin beta (β -actin) shown in Table S3. Because VKORC1 of chickens
277 and turkeys could not be cloned using the same primers as for quantitative real-time PCR,
278 VKORC1 was cloned using the alternative primers. The PCR was performed using
279 SapphireAmp® (Takara, Tokyo, Japan). The PCR cycle program was on one cycle of 30
280 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
281 extension for 1 min at 72°C.

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

285

286 *Quantitative real-time PCR*

287 Gene-specific quantitative real-time PCR primers (Table S3) were synthesized
288 by Sigma- Aldrich (Tokyo, Japan). The efficiency of all primers was 89%–107%.
289 Quantitative real-time PCR (qPCR) was performed with the StepOnePlus Real-Time PCR
290 system (Applied Biosystems, Foster City, CA). The 10 μ l PCR reaction mixtures
291 consisted of the Fast SYBR Green Master Mix (Applied Biosystems), forward and
292 reverse primers (200 nM each), and cDNA derived from 80 ng of total RNA. Plasmids
293 containing each amplicon were used for the calibration curves. All samples, including
294 cDNA derived from the tissues of chickens and turkeys and the plasmid standards, were
295 analyzed in duplicate using the following protocol: 95°C for 20 s followed by 40 cycles
296 of 95°C for 3 s and 60°C for 30 s. At the end of each PCR run, melt curve analysis was
297 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).

316 **RESULTS**

317 *Comparison of VKOR activity among avian species*

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

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

336

337 *Comparison of VKOR activity inhibited by warfarin among avian species*

338 Fig. 1A shows VKOR activity without warfarin, and VKOR activity with 1 or
339 10 μM warfarin incubation at 100 μM of VKO. With 1 μM warfarin, VKOR inhibition
340 did not occur in chickens and turkeys. However, VKOR inhibition was found in other
341 avian species. With 10 μM warfarin, inhibition of VKOR activity occurred in every avian
342 species. For male chickens (n=3), VKOR activity without warfarin, with 1 μM warfarin,
343 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 μ M
345 warfarin, and with 10 μ M 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 μ M warfarin
355 incubation compared to VKOR activity of untreated microsomes. For male and female
356 chickens, and turkeys, 1 μ M warfarin inhibited control activity by $106 \pm 4.6\%$ and $106 \pm$
357 16% , and $93 \pm 1.7\%$, respectively. For *Accipiter* (a goshawk and sparrowhawks) and *Bubo*
358 (snowy owls and great horned owls), 1 μ M warfarin inhibited control activity by $80 \pm$
359 13% and $79 \pm 11\%$, respectively. 1 μ M 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 μ M warfarin inhibited control activity by 85% and 45%,
362 respectively.

363 For male and female chickens, and turkeys, 10 μ M warfarin inhibited control
364 activity by $69 \pm 8.8\%$, $75 \pm 16\%$, and $53 \pm 2.2\%$, respectively. For *Accipiter* (a goshawk
365 and sparrowhawks) and *Bubo* (snowy owls and great horned owls), 10 μ M warfarin
366 inhibited control activity by $60 \pm 13\%$ and $38 \pm 15\%$, respectively. 10 μ M 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 μ M warfarin inhibited
369 control activity by 62% and 12%, respectively.

370

371 *Amino acid sequence alignment of VKORC1 and VKORC1L1 in avian species*

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

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

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

397 Few studies have reported the amino acid mutations of VKORC1L1 that
398 contribute to VKOR activity. Hünérberg (2009) used HEK cells expressing recombinant
399 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).

426 **DISCUSSION**

427 **1. Avian interspecific differences in VKOR activity**

428 *1-1. Comparison of VKOR activity among avian species*

429 This study suggested hepatic VKOR activities of raptors were lower than that
430 of turkeys and chickens. VKOR plays an important role in the vitamin K cycle for
431 activations of vitamin K-dependent coagulation factors. Inhibiting VKOR by ARs
432 induces lethal hemorrhages in raptors (Murray, M, 2018; Rattner et al., 2012; Rattner et
433 al., 2014; Rattner et al., 2015). Because the present study focused on comparing the
434 maximum VKOR activity between avian species, 12.5, 100, and 400 μM of VKO were
435 used as substrates for 1.0 mg/mL liver microsomes. The estimated concentration of VKO
436 is 12.5, 100, and 400 $\mu\text{mol/g}$ liver microsomes, respectively. However, the physiological
437 concentration of VKO is approximately 13 pmol/g in chicken liver (Will et al., 1992) and
438 0.1 pmol/g in mice liver (Okano et al., 2008). The concentration of VKO used for the
439 VKOR activity assay in the present study is much higher than physiological
440 concentrations. It is also important to investigate the activity under physiological
441 concentrations of the substrate.

442 It is reported that concentration of vitamin K in liver of chickens is less than
443 that of rats, while the concentration of VKO in liver is higher in chickens than in rats
444 (Will et al., 1992). This indicates that chickens have a poor ability to reduce VKO in the
445 vitamin K cycle, so they require high doses of vitamin K in their diets. Similarly, it is
446 possible that raptors require high doses of dietary vitamin K. K vitamins are divided into
447 two major types. Vitamin K₁ (phylloquinone) is synthesized in plants and is the primary
448 dietary source of vitamin K. Vitamin K₂ (menaquinones, MK) represents a family of
449 many different subtypes. One subtype, MK₄, is converted from phylloquinone and is the
450 major physiological vitamin K form (Van Horn 2013). Although many other
451 menaquinones are converted by gut bacteria, the menaquinones of gut origin make a
452 relatively minor contribution to the hepatic stores of vitamin K. Phylloquinone and MK₄
453 are also available from meat, including rats and mice, which are diets of raptors (Will et

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

457

458 *1-2. VKOR activity related to amino acid sequence and mRNA expression of VKORC1*
459 *and VKORC1L1*

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

474 Although it is difficult to compare the absolute amount of VKORC1 and
475 VKORC1L1 between turkeys and chickens, the total expression amount of VKORC1 and
476 VKORC1L1 was 2.5-fold higher in turkey liver (total amount was 3050, VKORC1 was
477 2700, and VKORC1L1 was 350 copy number/ng total RNA) than in chicken liver (total
478 amount was 1200, VKORC1 was 500, and VKORC1L1 was 700 copy number/ng total
479 RNA). The total expression amount of VKORC1 and VKORC1L1 could also cause avian
480 interspecific differences in VKOR activity. Information on the mRNA expression ratio
481 and the total quantities of VKORC1 and VKORC1L1 from various avian species might

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

483

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

485 *2-1. Comparison of VKOR activity inhibited by warfarin among avian species*

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

498

499 *2-2. Amino acid sequence of VKORC1*

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

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

571

572 **CONCLUSIONS**

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

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588

589

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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)	<i>Gallus gallus domesticus</i>	Male	3	a
		Female	3	a
Turkey (Bronze)	<i>Meleagris gallopavo</i>	Male	3	b
Canary (Lemon)	<i>Serinus canaria</i>	Male	6	c
Peregrine falcon	<i>Falco peregrinus pealei</i>	Female	1	d
Sparrowhawk	<i>Accipiter nisus nisosimilis</i>	Female	2	d, e
Goshawk	<i>Accipiter gentilis fujiyamae</i>	Female	1	d
Steller's sea eagle	<i>Haliaeetus pelagicus</i>	Male	1	d
White-tailed eagle	<i>Haliaeetus albicilla</i>	Female	1	d
Snowy owl	<i>Bubo scandiacus</i>	Male	2	e
Great horned owl	<i>Bubo virginianus</i>	Male	1	e
		Female	1	e
Night heron	<i>Nycticorax nycticorax</i>	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 μ M 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 \pm S.D.

613

VKO [μ M]	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 \pm 22	78 \pm 28	49 \pm 31	38	38 \pm 15		26 \pm 3		9
100				79, 72	89, 70	44, 79	38	33, 38	
	222 \pm 36	124 \pm 50	97 \pm 66	75	70 \pm 19		36 \pm 3		14
400				77, 94	100, 94	52, 84	42	42, 51	
	270 \pm 17	153 \pm 57	103 \pm 65	85	83 \pm 21		45 \pm 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.

617 Table S1. Primers for avian VKORC1L1 cloning.

618 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	

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

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

622 * 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 _ for cloning	Forward	GTCGGGACAGCGCCATCAACGT	215	Not tested	NM_206807	This study
	Reverse	GTTGACGTAGGTGCTGAG				
Chicken_ VKORC1L1	Forward	AGAAGGGCCGCGATCTCCACTACCA	106	89.2	NM_001001328	This study
	Reverse	CCCAACAGACCGAATCCTCGACCCCAT				
Chicken_GAPDH	Forward	CTCTGTTGTTGACCTGACCT	125	98.8	NM_204305	Watanabe et al., 2013
	Reverse	CAACCTGGTCCTCTGTGTAT				
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_ for cloning	Forward	GTTTGTGGGTCGGGACAGCGCCA	219	Not tested	XM_010726800	This study
	Reverse	ACGGCGTAGGTGCTGAGGCAGA				

Turkey_	Forward	GAAGGGGCCGCGATCTCCACTACCA	106	104.0	XM_003211735	This study
VKORC1L1	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				

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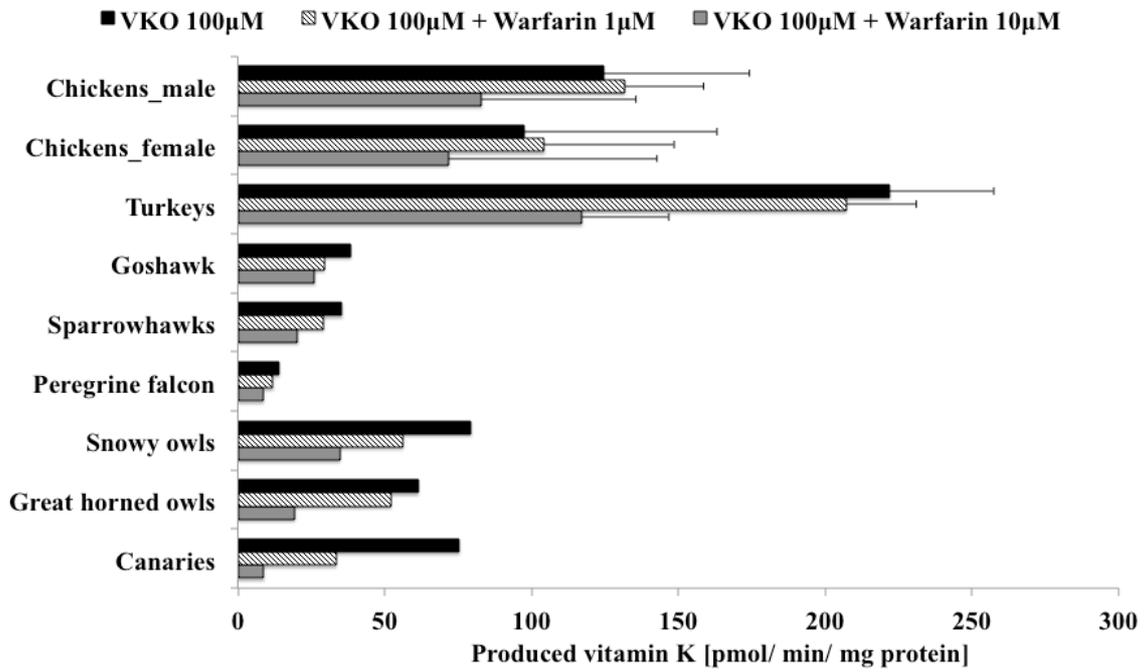
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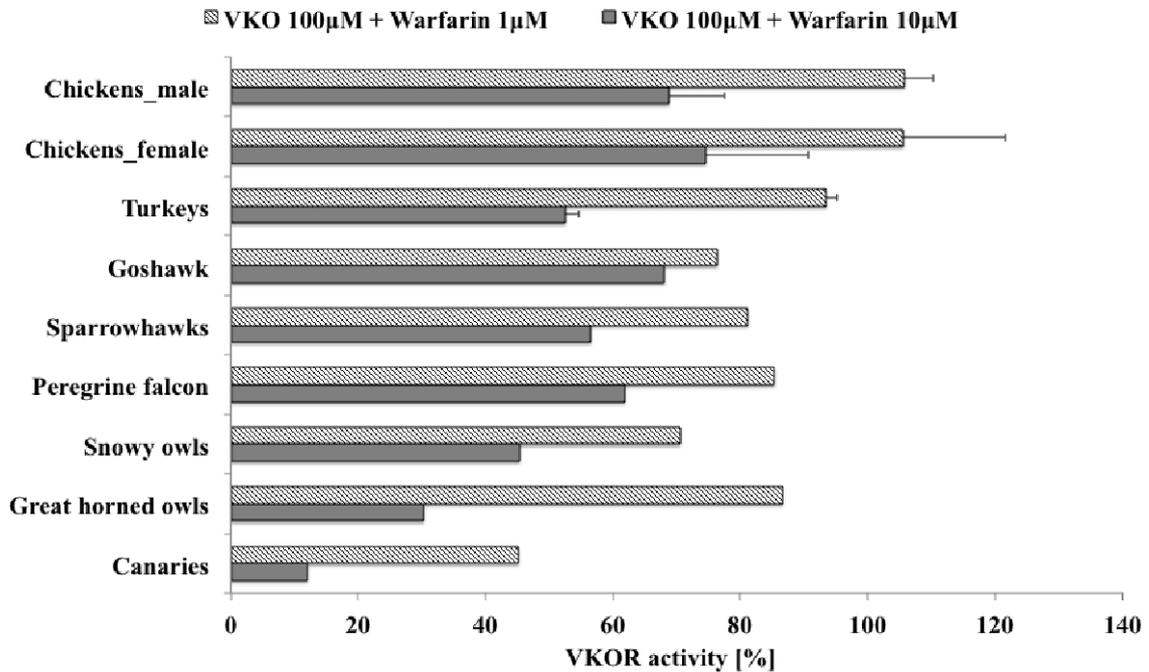
637



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

645 raptors.

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

647 $\text{VKOR activity (\%)} = \text{VKOR activity with warfarin} / \text{that without warfarin} * 100$

648 Each data point from turkeys and chickens represents the mean of three animals \pm S.D.

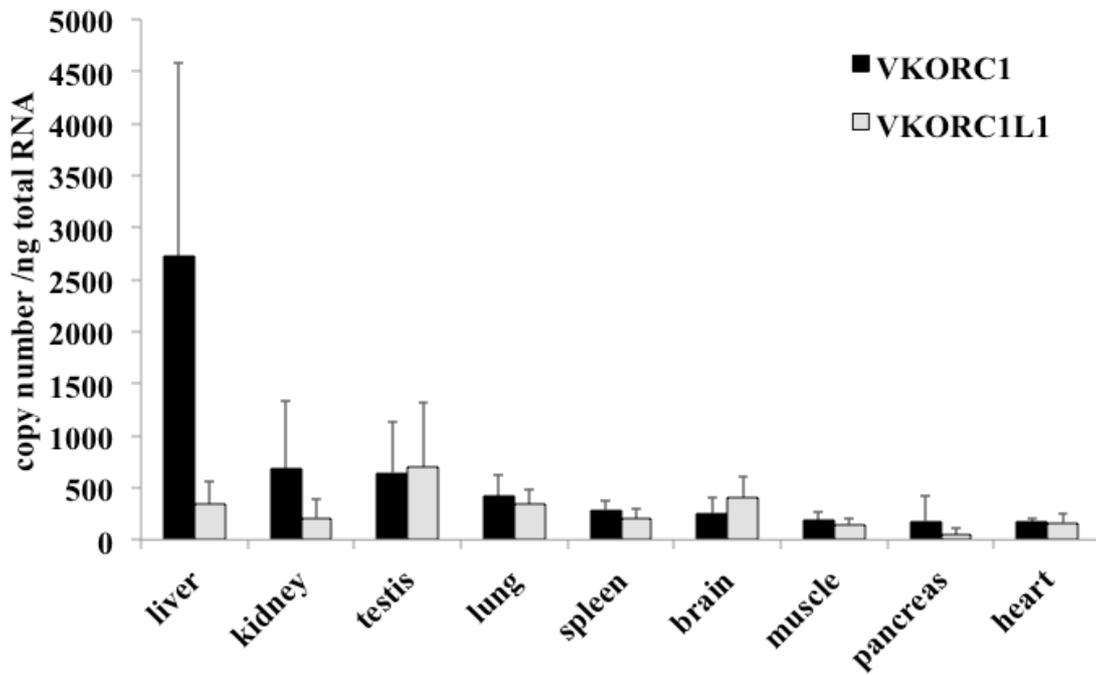
649 (error bars). The numbers of other species were less than three. Therefore there are no

650 S.D. values. No significant differences in percentage of VKOR activity at each warfarin

651 concentration among chickens, *Accipiter*^a (a goshawk and sparrowhawks), turkeys and

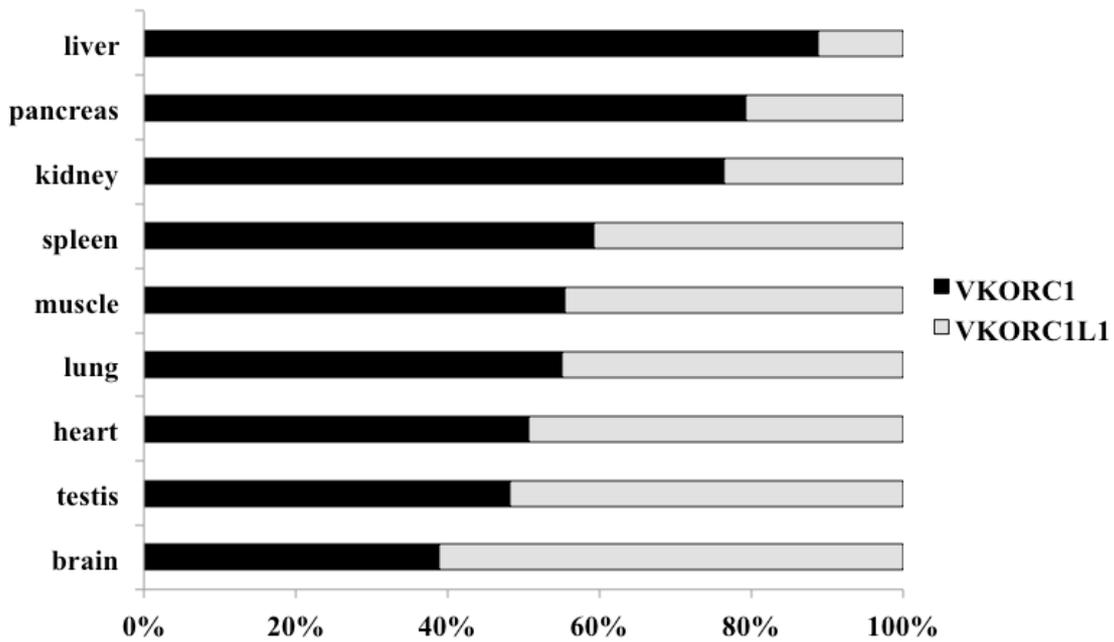
652 *Bubo*^b (snowy owls and great horned owls) were observed (Steel-Dwass test, $P < 0.05$).

653 A



654

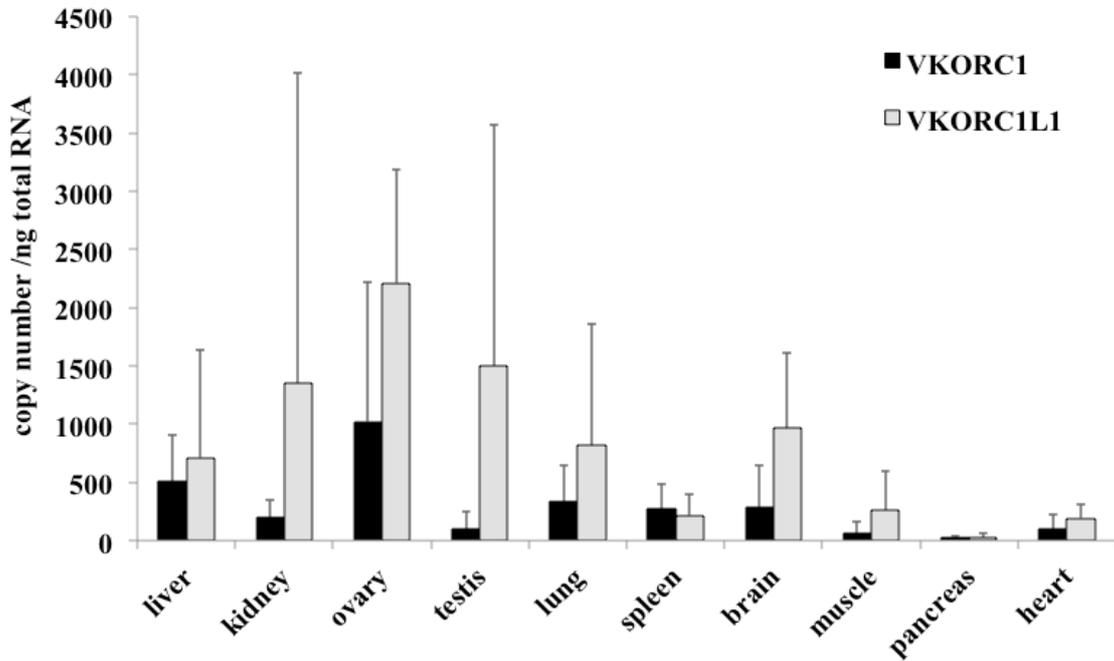
655 B



656

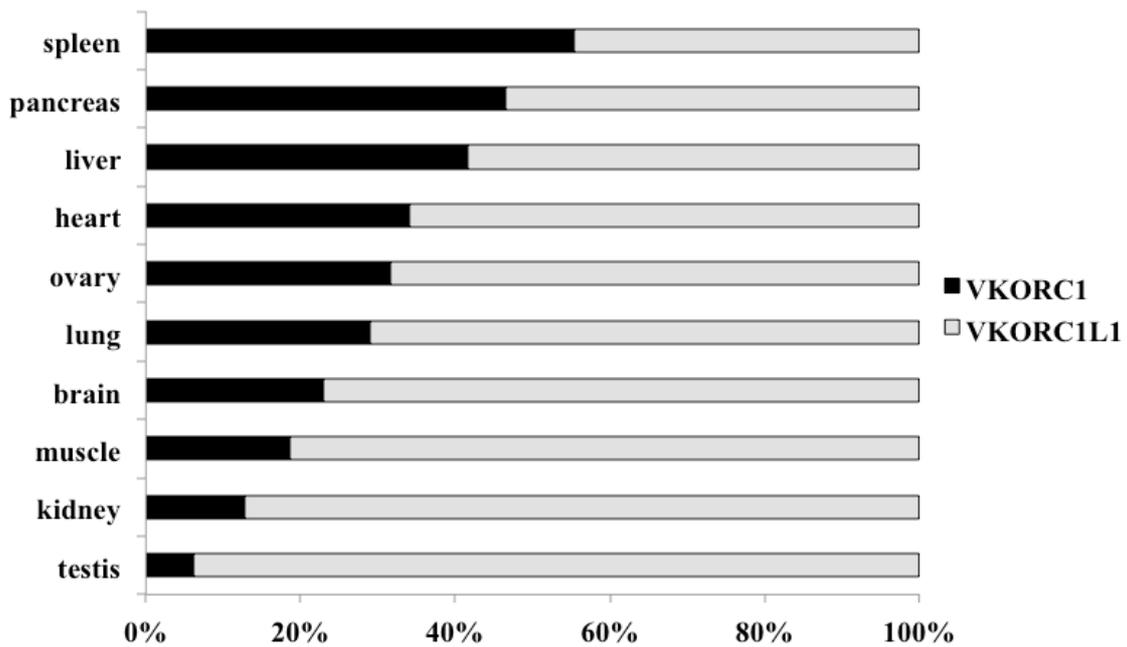
657 Fig. 2. mRNA expression of VKORC1 and VKORC1L1 (A) and their ratios (B) in nine
658 different tissues of male turkeys. Each data represents the mean of three animals \pm S.D.
659 (error bars).

660 A



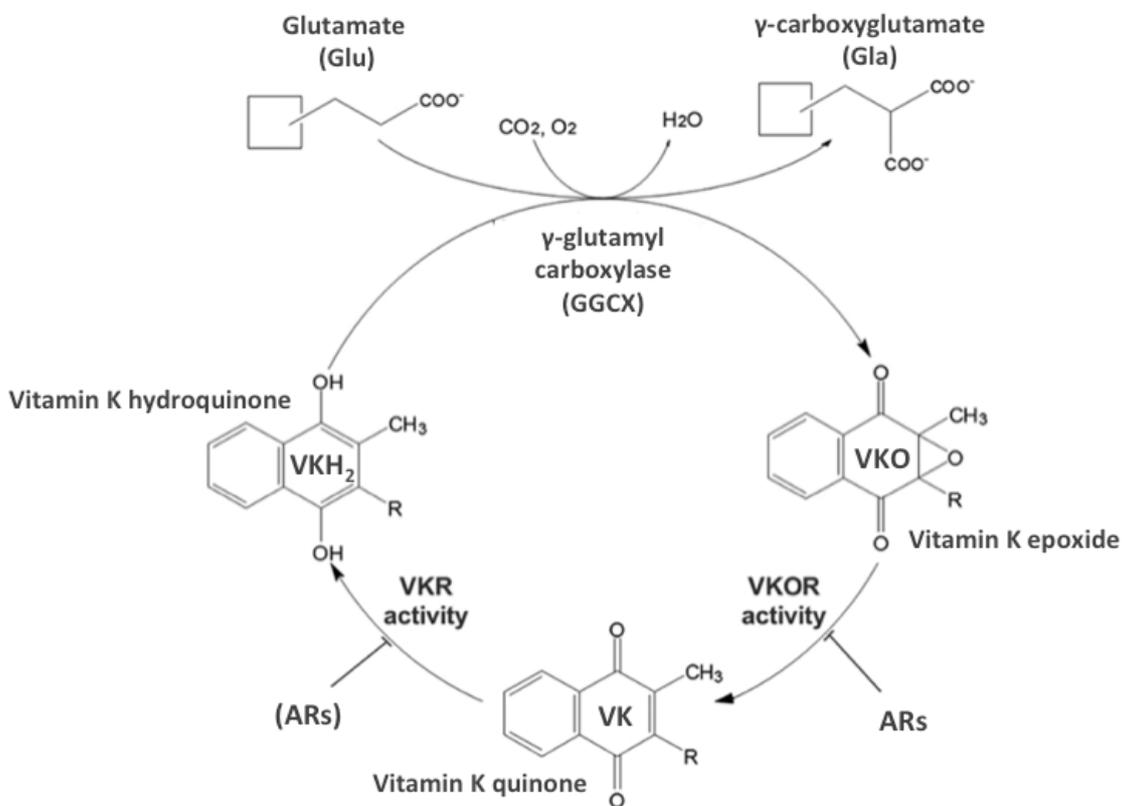
661

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 \pm S.D. (error bars) except for ovary and testis. Data of ovary and testis
667 represents the mean of three animals \pm S.D. (error bars).



668

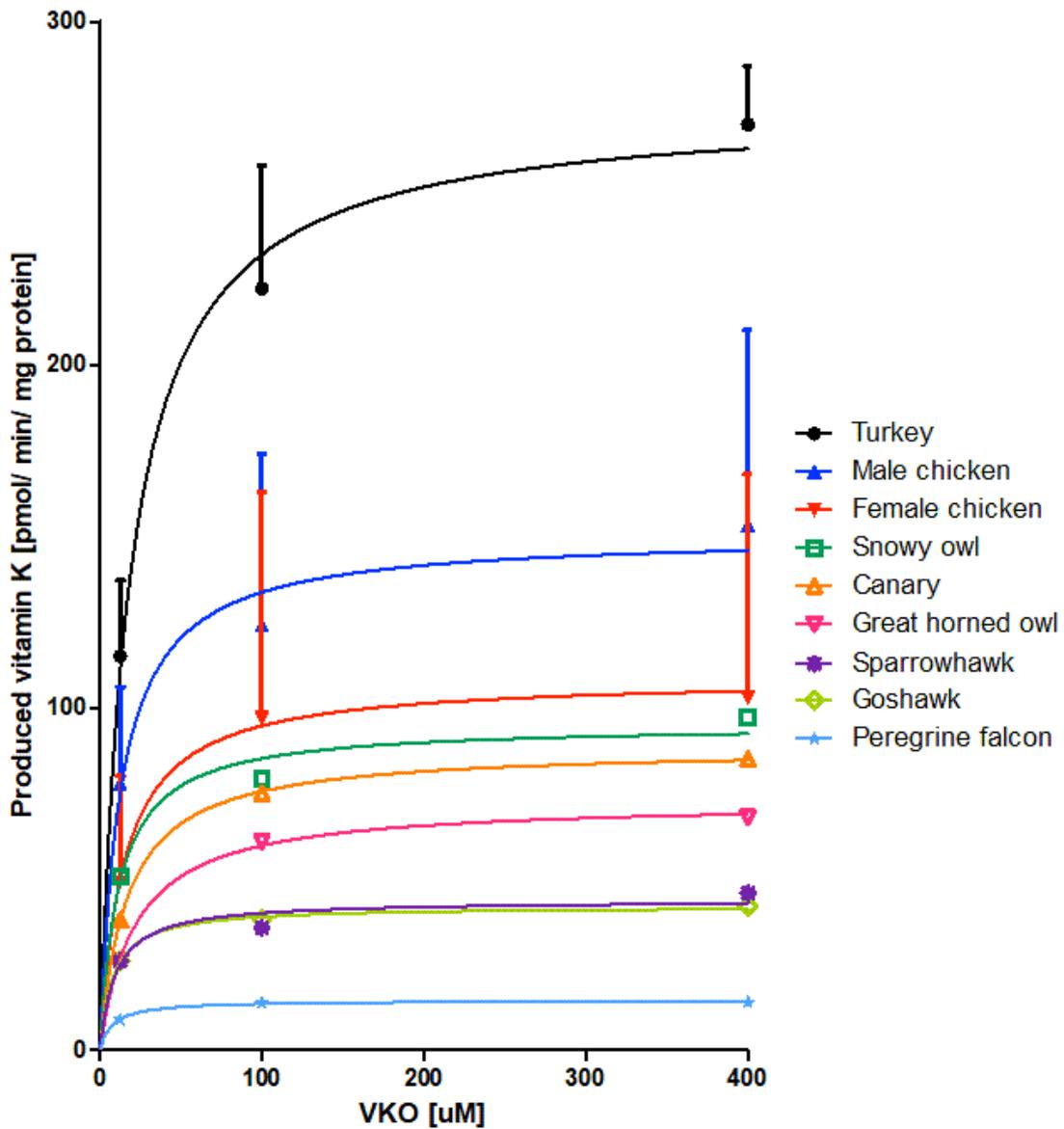
669 Fig. S1. Modified vitamin K cycle (Hammed et al., 2013; Tie et al., 2013).

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

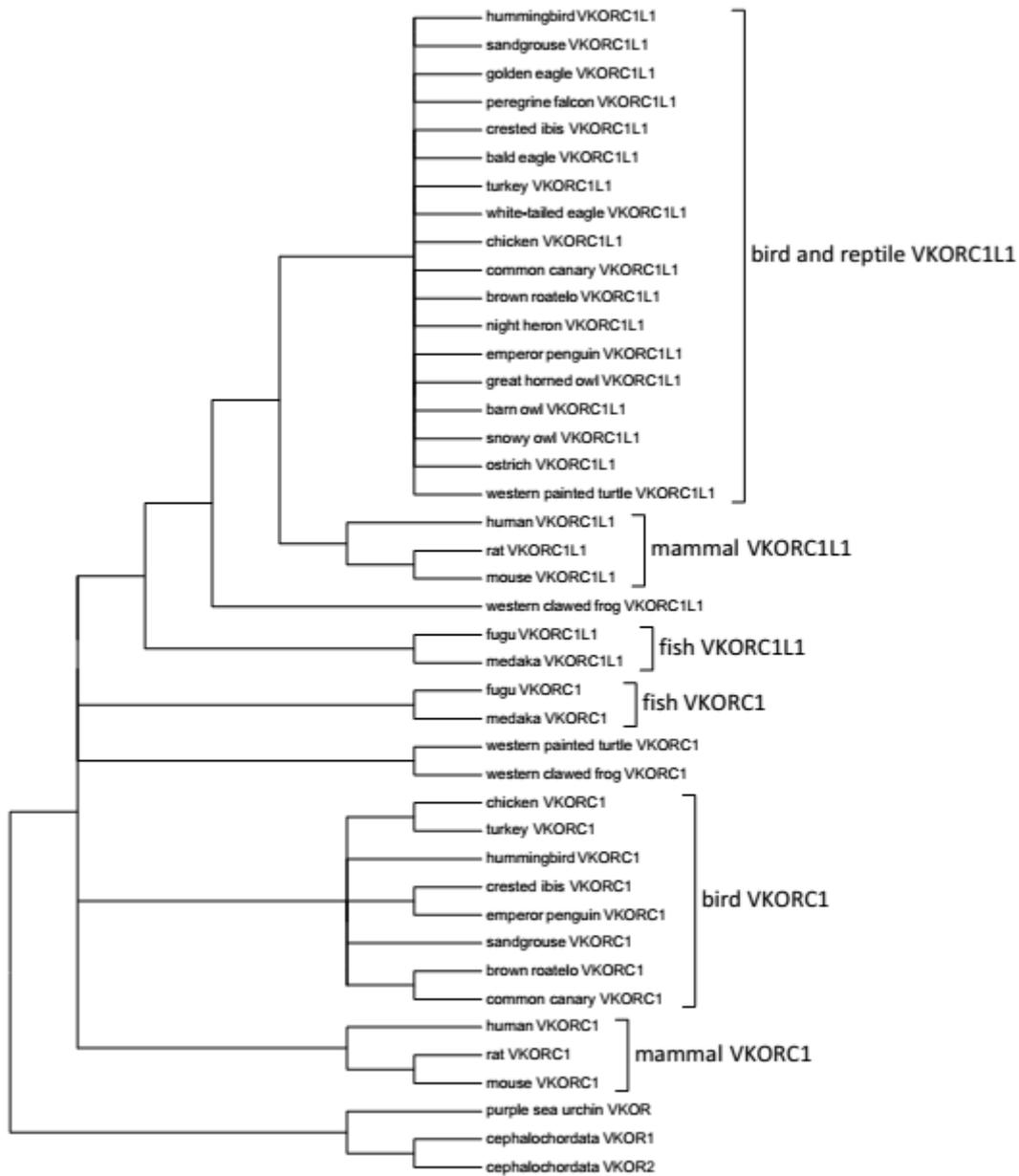


674

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 \pm S.D. (error bars). The numbers of other species were less than three, therefore
 680 there are no S.D. values.

681



682

683 Fig. S3. Phylogenetic tree of the VKOR protein family. In all vertebrates, VKORC1L1

684 made one cluster. VKORC1 were collapsed because of low bootstrap value (less than

685 60%). In VKORC1, 4 clusters; fish, bird, mammal, and other animal VKOC1, were made.

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

	1			36					
human_C1L1	MAAPVLLRVS	VPRWERVARY	AVCAAGILLS	IYAYHVEREK	ERDPEHRALC	DLGPPWVKCSA	ALASRWGRGF	GLLGSIFGKD	GVLNQPNVSF
rat_C1L1	MAAPVLLRVS	VPRWERVARY	AVCAAGILLS	IYAYHVEREK	ERDPEHRALC	DLGPPWVKCSA	ALASRWGRGF	GLLGSIFGKD	GVLNQPNVSF
mouse_C1L	MAAPVLLRVS	VPRWERVARY	AVCAAGILLS	IYAYHVEREK	ERDPEHRALC	DLGPPWVKCSA	ALASRWGRGF	GLLGSIFGKD	GVLNQPNVSF
ostrich_C1L1	-----	-----	-----	-----	-----	-----	----RWGRGF	GLLGSIFGKD	SAINQPNVSF
chicken_C1L1	MAAPVLLRVS	VPRWERVARS	AVCAAGILLS	LYACHLEREK	GRDLHYQALC	DLSEVRCSA	AITSRWGRGF	GLLGSIFGKD	SAINQSNVSF
turkey_C1L1	MAAPVLLRVS	VPRWERVARS	AVCAAGILLS	LYACHLERRR	GRDLHYQALC	DLSEVRCSA	AITSRWGRGF	GLLGSIFGKD	SAINQSNVSF
hummingbird_C1L1	-----	-----	-----	-----	-----	-----	----RWGRGF	GLLGSIFGKD	SAINQSNVSF
broen_roateLo_C1L1	-----	-----	-----	-----	-----	-----	----WGRGF	GLLGSIFGKD	SAINQSNVSF
sandgrouse_C1L1	-----	-----	-----	-----	-----	-----	----RWGRGF	GLLGSIFGKD	SAINQSNVSF
crested_ibis_C1L1	-----	-----	-----	-----	-----	-----	----RWGRGF	GLLGSIFGKD	SAINQSNVSF
night_heron_C1L1	-----	-----	-----	-----	-----	-----	-----	-----	-----
emperOr_penguin_C1L1	-----	-----	-----	-----	-----	-----	----RWGRGF	GLLGSIFGKD	SAMNQSNVSF
golden_eagle_C1L1	MAAPVLLRVS	VPRWERVARS	AVCAAGILLS	LYACHLEREK	GRDLHYQALC	DLSEVRCSA	AITSRWGRGF	GLLGSIFGKD	SAINQSNVSF
bald_eagle_C1L1	MAAPVLLRVS	VPRWERVARS	AVCAAGILLS	LYACHLEREK	GRDLHYQALC	DLSEVRCSA	AITSRWGRGF	GLLGSIFGKD	SAINQSNVSF
white_tailed_eagle_C1L1	-----	-----	-----	-----	-----	-----	----WGRGF	GLLGSIFGKD	SAINQSNVSF
barn_owl_C1L1	-----	-----	-----	-----	-----	-----	----RWSRGF	GLLGSIFGKD	SAVNQSNVSF
snowy_owl_C1L1	-----	-----	-----	-----	-----	-----	-----	-----	-----
great_horned_owl_C1L1	-----	-----	-----	-----	-----	-----	-----	-----	-----
peregrine_falcon_C1L1	-----	-----	-----	-----	-----	-----	----RWGRGF	GLLGSIFGKD	SAINQSNVSF
canary_C1L1	MAAPVLLRVS	VPRWERVARS	AVCAAGILLS	LYACHLEREK	GRDSHYQALC	DLSEVRCSA	AITSRWGRGF	GLLGSIFGKD	SAINQSNVSF
turtle_C1L1	MAAPVLLRVS	VPRWERVARY	VVCAAGILLS	LYACHLEREK	GLDLHYRALC	DISERVCSA	AIASRWGRGF	GLLGSIFGKD	SAINQPNVSF
frog_C1L1	MAAPVM-RVS	VPRWESGARY	AVCVLGIVLS	IYAFHVEREK	ERDPGYKAIC	DFNEWVHCST	VLSRWGRGF	GMLGSIFGKD	SLLNQPNVSF
fugu_C1L1	MAAPVL-RVS	TPRWERIARV	LVCLLGILLS	LYAFHVEREH	ARDPSYKALC	DVSSSISCSK	VFGSRWGRGF	GLLGSIFGND	SALNQPNSVY

	91					147			
human_C1L1	GLIFYILQLL	LGMTASAVAA	LILMTSSIMS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	LLLIINYKRL	VYLNEAWKRQ	LQPKQD*
rat_C1L1	GLIFYILQLL	LGMTASAVAA	LVLMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVTITYLLNF	LLLIINYKRL	VYLNEAWKRQ	LQPKED*
mouse_C1L	GLIFYILQLL	LGMTASAVAA	LVLMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVTITYLLNF	LLLIINYKRL	VYLNEAWKRQ	LQPKED*
ostrich_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
chicken_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
turkey_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
hummingbird_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
broen_roateLo_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
sandgrouse_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
crested_ibis_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
night_heron_C1L1	-----	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	L-----
emperOr_penguin_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
golden_eagle_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
bald_eagle_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
white_tailed_eagle_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
barn_owl_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
snowy_owl_C1L1	-----	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	L-----
great_horned_owl_C1L1	-----	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	L-----
peregrine_falcon_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
canary_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
turtle_C1L1	GLVFYILQML	LGMTASAVAA	LILMTSSIVS	VVGSLYLAYI	LYFVLKEFCI	ICVITYLLNF	ILFIINYKRL	VYLNEAWKRQ	LQPKQE*
frog_C1L1	GLVFYLLQML	LGMTVSAAVA	LVLMTSSIVS	VVGSVYLAYI	LYFVLKDFCV	ICVTITYLLNF	ILLIINYKRL	VYLNEAWKRQ	LQDKQE*
fugu_C1L1	GIVFYAFQLL	LGMTVSAMAA	LILMTSSIMS	VVGSVYLYGI	LYFVLKDLCV	ICVTITYLLNF	ILFVLYNYKRL	VYLNEAWKRQ	LQAKQD*

694 Fig. S4-B. Amino acid sequence alignment of VKORC1L1 of different species.
695 Grey box indicates the catalytic CXXC motif, which is supposed to be the active site of the VKORC1L1 protein. White box indicates the
696 warfarin-binding site. Brown or sky blue shading highlights amino acids whose mutations give warfarin resistance or low VKOR activity,
697 respectively (Hünerberg, 2009).

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