Characterization of function and genetic feture of UDP-glucuronisyltransferase in avian species

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ABSTRACT

Birds are exposed to many xenobiotics during their lifetime. For accurate prediction of xenobiotic-induced toxic effects on avian species, it is necessary to understand metabolic capacities in a comprehensive range of bird species. However, there is a lack of information about avian xenobiotic metabolizing enzymes (XMEs), particularly in wild birds. Uridine diphosphate glucuronosyltransferase (UGT) is an XME that plays an important role in phase II metabolism in the livers of mammals and birds. This study was performed to determine the characteristics of UGT1E isoform in avian species, those are related to mammals UGT 1A. To understand the characteristics of avian UGT1E isoforms, *in vitro* metabolic activity and genetic characteristics were investigated. Furthermore, mRNA expression levels of all chicken UGT1E isoforms were measured. On *in vitro* enzymatic analysis, the white-tailed eagle, great horned owl, and Humboldt penguin showed lower UGT-dependent activity than domestic birds. In synteny analysis, carnivorous birds were shown to have fewer UGT1E isoforms than herbivorous and omnivorous birds, which may explain why they have lower *in vitro* UGT activity. These observations suggested that raptors and seabirds, in which UGT activity is low, may be at high risk if exposed to elevated levels of xenobiotics in the environment. Phylogenetic analysis suggested that avian UGT1Es have evolved independently from mammalian UGT1As. We identified the important UGT isoforms, such as UGT1E13, and suspected their substrate specificities in avian xenobiotic metabolism by phylogenetic and quantitative real-time PCR analysis. This is the first report regarding the genetic characteristics and interspecies differences of UGT1Es in avian species.

Key Words:

UGTs; xenobiotic; bird; wildlife; toxicology

1. Introduction

Birds are exposed to a variety of xenobiotics in the wild, such as drugs, pesticides, and heavy metals (Donald et al., 1983; Alessandra et al., 2005; Fabricio et al., 2007), and many toxic effects of these agents have been reported. For example, in the Great Lakes of the USA, various carnivorous birds (meet-eating and fish-eating birds) showed increased rates of reproductive injury and adult mortality associated with exposure to dichlorodiphenyltrichloroethane (DDT) in the 1990s (Bowerman et al., 1995). On the Indian Peninsula, diclofenac caused a sharp decline in vulture numbers (Oaks et al., 2004). In addition, rodenticides, such as warfarin and brodifacoum, are responsible for secondary poisoning of birds around the world (Erickson et al., 2004). Thus, it is obvious that such chemicals influence avian reproduction and life. However, there have been no accurate predictions of toxicological effects on non-target species, and only a few studies have focused on the chemical sensitivity of various organisms. Therefore, it is necessary to determine chemosensitivity in avian species to achieve accurate toxicological evaluation.

Chemical behavior is represented as ADME (Absorption, Distribution, Metabolism, Excretion), all of which determine chemical sensitivity. Among ADME, metabolism is the most important factor for prediction of chemical sensitivity and it involves catalysis by many xenobiotic metabolizing enzymes (XMEs).

Chemicals are metabolized mainly in the liver by phase I and phase II enzyems. Phase I enzymes include primarily the cytochrome P450 (CYP) superfamily, whereas phase II conjugating enzymes include many enzyme super families such as uridine diphosphate-glucuronosyltransferase (UGT), sulfotransferase, and glutathione S-transferase (GST) (Xu et al. 2005). Some xenobiotics are metabolically activated by CYP and the resulting intermediates cause health dysfunction. The conjugation reaction is essential for detoxification (Kakehi et al. 2015). Especially, UGT play a major role in the elimination of nucleophilic metabolites of carcinogens, such as phenols and quinoles of polycyclic aromatic hydrocarbons. In humans, 55% of the 200 most frequently prescribed drugs are conjugated by UDP-glucuronosyltransferase (UGT) and eliminated in the urine or bile, which shows UGT is involved in the metabolism more substances than other phase II conjugation enzymes (Guillemette et al., 2014). Furthermore, UGT conjugates many endogenous substrates, including bilirubin, steroid hormones and thyroid hormone. (Matern et al., 1994). UGT catalyzes conjugation of the glycosyl group of glucuronic acid to lipophilic endogenous and exogenous substrates (Robert et al., 2000). The UGTs form a superfamily, which is further subdivided into four major families: UGT1, UGT2, UGT3, and UGT8 in mammals (Burchell 1991). Among these subfamilies, UGT1A and UGT2B subfamilies mainly contribute to liver drug metabolism in mammals. Based on sequence similarity and substrate specificity, the mammalian UGT1A genes can be divided into two groups, i.e., the bilirubin group (UGT1A1

through 1A5) and the phenol group (UGT1A6 through 1A10) (Ikushiro et al., 1995; Owens et al., 2005; Zhang et al., 2004). In almost all mammals, the UGT1 locus consists of variable first exons and four shared exons in a tandem array. Each variable exon encodes different polypeptides that form the substrate-binding domain. The four-shared exons encode the common C-terminal domain that binds to uridine diphosphoglucuronic acid (UDPGA). Each first exon possibly determines substrate specificity, whereas the common exons most likely determine the interaction with the common substrate, UDPGA. UGT1 genes have broad and overlapping substrate specificity due to the highly variable structure of their protein products (Robert et al., 2000). There are large interspecies differences in UGT1A metabolism due to this complex molecular diversity. For example, cats show high sensitivity to acetaminophen and acetylsalicylic acid (Savides et al., 1984) because UGT1A6, which contributes to glucuronidation of phenolic substrates in other mammals, is a pseudogene in this species (Court et al., 2000). Also in birds, interspecies differences of UGT activities might lead to sensitivity to some substances.

The avian UGT1 subfamily was designated as UGT1E by the UGT nomenclature committee depends on the similarity of sequence (http://prime.vetmed.wsu.edu/resources/udp-glucuronsyltransferase-homepage). Recently the evolutionary relationship of UGT1E genes was reported and it also suggested UGT1Es would be related to metabolizing exogenous compounds (Kawai et al., 2018 accepted). Moreover, some studies have already demonstrated the *in vitro* UGT activity in limited avian species. Zoltan et al. (1983) reported that avian UGT is involved in glucuronidation of exogenous compounds as in mammalian species. Saengtienchai et al. 2018 also compared hepatic microsomes UDP-glucuronosyltransferase activity toward 1-hydroxypyrene in rats, chickens and quails, and found that the avian Vmax/Km was lower than that of rats in vitro. We considered that this discrepancy may be explained by the potentially divergent biotransformation in birds and rats due to differences in the pattern of xenobiotic elimination. However, there have been few studies regarding glucuronidation activity in wild birds. The present study was performed to determine the characteristics of UGT1E in avian species, including wild birds. First, *in vitro* UGT activity in seven avian species, including wild birds, and in rats liver microsomes were measured and compared. The genetic characteristics of avian UGT1E isoforms were also investigated. Synteny and phylogenetic analyses were performed to compare each UGT1E isoform in several avian species that were used in activity study and in their related species. Quantitative real-time polymerase chain reaction (qPCR) was also performed to investigate the mRNA expression levels of nine chicken UGT1E isoforms.

This is the first comprehensive report regarding interspecies differences in UGT glucuronidation activities and their genetic characteristics of avian UGT1E isoforms.

2. Materials and methods

All animal experiments were performed under the supervision and with the approval of the Institutional Animal Care and Use Committee of Hokkaido University, Japan (approval number 14-0119), which conform to the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

Chemicals. 1-Hydroxypyrene, pyrene glucuronide, acetaminophen glucuronide, β-estradiol, sodium cholate hydrate, UDPGA, and RNA later ® were obtained from Sigma-Aldrich (St. Louis, MO). Acetaminophen, acetic acid, sodium phosphate, and ammonium acetate solution were purchased from Wako Pure Chemical Industries Ltd. (Osaka, Japan). β-Estradiol-3-β-*D*-glucuronide sodium salt was obtained from Santa-Cruz Biotechnology Inc. (Santa-Cruz, CA). All chemicals used for high-performance liquid chromatography (HPLC) and mass spectrometry (MS) were HPLC or MS grade and were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan).

2.1. Animals

Various avian species were selected and used for experiments in feeding habits and phylogenetic tree. Liver samples were collected from chickens (*Gallus gallus domesticus*), turkeys (*Meleagris gallopavo*), ostriches (*Struthio camelus*), canaries (*Serinus canaria*), white-tailed eagles (*Haliaeetus albicilla*), a great horned owl (*Bubo virginianus*), a Humboldt penguin (*Spheniscus humboldti*), and rats (*Rattus norvegicus*; Sprague–Dawley strain) as shown in Supplementary Table S1. All animals were male. Chickens (13 months old) were provided by the farm at Hokkaido University (Sapporo, Hokkaido, Japan). Turkeys (20 months old) were purchased from Sankyo Labo Service Corporation, Inc. (Tokyo, Japan). Canaries (6 months old) were purchased from a local pet shop (Sapporo, Hokkaido, Japan). Turkeys and canaries were acclimatized to the environment maintained at a constant temperature (22°C ± 1°C), constant humidity (55% ± 5%), and with a 12:12 hour light:dark cycle, with food and water *ad libitum* for 1 week before commencement of the experiment. Chickens, turkeys, and canaries were anesthetized using isoflurane (DS Pharma Animal Health, Osaka, Japan) and euthanized by CO_2. The liver of the great horned owl (2 years old; cause of death, unknown) and Humboldt penguin (1 year old; died of respiratory disease) were provided by Sapporo Maruyama Zoo (Sapporo, Hokkaido, Japan). The livers of white-tailed eagles (< 3 years old; died in traffic accidents) were provided by the Institute for Raptor Biomedicine Japan (Kushiro, Hokkaido, Japan). The livers of ostriches (< 2 years old) were provided by Misato Ostrich Farm (Misato, Hokkaido, Japan). Sprague–Dawley rats were purchased from Sankyo Labo Service Corporation, Inc. Rats at 8 weeks old were used for comparison. The rats (7 weeks old) were housed under conditions of constant temperature (23°C ± 1°C), constant humidity (55% ± 5%),

and automatically controlled lighting (07:00−19:00) with food and water *ad libitum* and handled for 1 week. After euthanasia of rats by CO_2, the livers were collected. All liver samples from the eight species were immediately frozen in liquid nitrogen and stored at –80°C until use.

2.2. Feeding habits of avian species

The feeding habits information of nine of the avian species used in UGT activity tests and genetic analyses were determined with reference to the report of Almeida et al. (2016). The feeding habits of the remaining three avian species (canary, great horned owl, and Japanese quail) were determined according to the reports of Goldsmith (1982), Paul et al. (1940), and Andrew et al. (1997), respectively. Based on these reports, we regarded the ostrich and canary as herbivorous species, the turkey, chicken, Japanese quail, and mallard as omnivorous birds, and the white-tailed eagle, bald eagle, peregrine falcon, great horned owl, Humboldt penguin, Adelie penguin, and Northern fulmar as carnivores.

2.3. Preparation of microsomes

Liver microsomes were prepared according to the method of Omura and Sato (1964). Samples of about 5 g of liver tissue from each of the seven avian species and Sprague–Dawley rats were homogenized in 15 ml of potassium phosphate buffer (KPB: 0.1 M, pH 7.4). Homogenates were transferred into tubes and centrifuged at $9000 \times g$ at 4°C for 20 minutes. The supernatants were further centrifuged at $105000 \times g$ for 70 minutes to obtain the microsomal fractions. Microsomal pellets were resuspended in 5 ml of buffer. Microsomes were stored at –80°C until analysis. The protein concentration of hepatic microsomes was measured using a BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA) according to the manufacturer's instructions. Microsomal concentrations of protein and CYP450 are shown in Supplementary Table S1.

2.4. Spectrum of cytochrome P450 (P450) for evaluation of microsomal quality

The quality of P450, which is one of the XMEs and plays a role in phase I metabolism, was investigated as an indicator of enzymatic condition. The reduced CO spectrum between 400–500 nm was examined by spectrophotometry (UV-2600; Shimadzu, Kyoto, Japan) according to the method of Omura and Sato (1964). The unique peak at 450 nm for P450 was checked to evaluate the quality of microsomal samples.

Defined peaks at 450 nm could be observed in all microsomal samples. Therefore, we regarded the quality of UGT in microsomes was conserved.

2.5. In vitro UGT activity tests

2.5.1. β-Estradiol glucuronidation assay

UGT activity of β-estradiol was assessed using the method described by Kakehi et al. (2015) with slight modifications. First, hepatic microsomal solution was mixed with KPB (0.1 M, pH 7.4) and prepared to 4 mg/ml. Aliquots of microsomal preparations (12.5 µl) were then mixed with 35 µl of KPB and 2.5 µl of 1% sodium cholate solution, and incubated on ice for 30 minutes. Then, 50 µl of microsomal solution was mixed with 41.5 µl of KPB (0.1 M, pH 7.4), 5 µl of 100 mM MgCl, and β-estradiol, which was dissolved in methanol, resulting in a final concentration of 1% methanol in a total volume of 97.5 µl. The final β-estradiol concentration was varied between 12.5 µM and 500 µM. Samples were then preincubated in a water bath for 5 minutes. The reaction was initiated by adding 2.5 µl of 100 mM UDPGA. After incubation for 15 minutes, the reaction was stopped by adding 200 µl of ice-cold methanol. The temperatures for preincubation and reaction were 41.5°C for turkey, 42°C for chicken, 38.5°C for canary and ostrich, 40.5°C for white-tailed eagle, 39.5°C for great horned owl, 39.0°C for Humboldt penguin, and 37.0°C for rat (McNab and Brian 1966; Siegfried et al., 1975; Chaplin et al., 1984; Herrero and Barja 1998; Richards 1971). Reaction mixtures were then placed on ice for 15 minutes before centrifugation at 15000 × *g* for 10 minutes. The resultant supernatants were injected into a liquid chromatography/mass spectrometry (LC/MS) system. The HPLC system (pump: LC-20AD; auto sampler: SIL-20A; column oven: CTO-20A; controller: CEM-20A; Shimadzu) coupled with electrospray ionization triple quadrupole mass spectrometry (ESI-MS/MS) (LCMS-8040; Shimadzu) was equipped with an Inertsil ODS-3 column (2.1 mm × 150 mm, particle size 5 µm; GL Sciences, Inc., Tokyo, Japan). The collision energies (CE) and other MS parameters were optimized and are shown in Supplementary Table S2. Mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0) and phase B consisted of phase A:acetonitrile (1:9 v/v). The solvent gradient was as follows: 5% mobile phase B from 1.5 to 7 minutes followed by a linear gradient to 95%, 95% mobile phase B from 7 to 8.5 minutes, and then 5% mobile phase B from 8.5 to 10 minutes. An injection volume of 5 µl, flow rate of 0.3 ml/min, and column temperature of 45°C were used.

2.5.2. 1-Hydroxypyrene glucuronidation assay

The UGT conjugating activity of 1-hydroxypyrene was assessed using the method described by Ueda et al. (2011) with slight modifications. First, hepatic microsomal solution was mixed with KPB (0.1 M, pH 7.4) and prepared to 4 mg/ml. Microsomal preparation (12.5 µl) was then mixed with 35 µl of KPB and 2.5 µl of 1% sodium cholate solution and incubated on ice for 30 minutes. Then, 50 µl of microsomal solution was mixed with 41.5 µl of KPB (0.1 M, pH

7.4), 5 µl of 100 mM MgCl, and 1-hydroxypyrene, which was dissolved in methanol, resulting in a final concentration of 1.0% methanol, in a total volume of 100 µl. The final 1-hydroxypyrene concentration was varied between 10 µM and 200 µM. Samples were then preincubated in a water bath for 5 minutes. The reaction was initiated by adding 5 µl of 100 mM UDPGA. After incubation for 10 minutes, the reaction was stopped by adding 400 µl of ice-cold methanol. The temperatures for preincubation and reaction were the same as in the β-estradiol glucuronidation assay. Reaction mixtures were then placed on ice for 15 minutes before centrifugation at 15000 × *g* for 10 minutes. The resultant supernatants were injected into an HPLC system. Analysis was performed on an HPLC system (pump: LC-20AB; auto sampler: SIL-20AC; column oven: CTO-20A; controller: CBM-20A; Shimadzu) using a fluorescence detector (FD) (SPD-20A; Shimadzu) equipped with an Inertsil ODS-3 column (2.1 mm × 150 mm, particle size 5 µm; GL Sciences, Inc.). Mobile phase A consisted of 10 mM ammonium acetate buffer (pH 5.0):acetonitrile (9:1, v/v) and phase B consisted of acetonitrile. The solvent gradient was as follows: 10% mobile phase B from 0 to 7 minutes followed by a linear gradient to 90% mobile phase B from 7 to 8 minutes, and then 10% mobile phase B from 8 to 10 minutes. An injection volume of 5 µl, flow rate of 0.3 ml/min, and column temperature of 45°C were used.

2.5.3. Acetaminophen glucuronidation assay

The UGT metabolic activity of acetaminophen was assessed using the method described by Kakehi et al. (2015) with slight modifications. First, hepatic microsomal solution was mixed with KPB (0.1 M, pH 7.4) and prepared to a microsomal concentration of 4 mg/ml. The microsomal preparations (12.5 µl) were then mixed with 35 µl of KPB and 2.5 µl of 1% sodium cholate solution and incubated on ice for 30 minutes. Then, 50 µl of microsomal solution was mixed with 41.5 µl of KPB (0.1 M, pH 7.4), 5 µl of 100 mM MgCl, and acetaminophen in a total volume of 97.5 µl. The final acetaminophen concentration was varied between 0.5 mM and 30 mM. Samples were then preincubated in a water bath for 5 minutes. The reaction was initiated by adding 5 µl of 100 mM UDPGA. After incubation for 15 minutes, the reaction was stopped by adding 200 µl of ice-cold methanol. The temperatures for preincubation and reaction were the same as in the β-estradiol glucuronidation assay. Reaction mixtures were then placed on ice for 15 minutes before centrifugation at 15000 × *g* for 10 minutes. The resultant supernatants were injected into an LC/MS system. An HPLC system (pump: LC-20AD; auto sampler: SIL-20A; column oven: CTO-20A; controller: CEM-20A; Shimadzu) coupled with electrospray ionization mass spectrometry (ESI-MS/MS) (LCMS-8040; Shimadzu) equipped with an Inertsil ODS-3 column (2.1 mm × 150 mm, particle size 5 µm; GL Sciences, Inc.) was used. The collision energies (CE) and other MS parameters were optimized and are shown in Supplementary Table S2. Mobile phase A consisted of 0.1% formic acid in DDW and phase B consisted of 0.1% formic acid in acetonitrile. The solvent gradient was as follows: 5% mobile

phase B from 1.5 to 7 minutes followed by a linear gradient to 95%, 95% mobile phase B from 7 to 8.5 minutes, and then 5% mobile phase B from 8.5 to 10 minutes. An injection volume of 5 µl, flow rate of 0.3 ml/min, and column temperature of 45°C were used throughout.

2.6. Phylogenetic analysis of UGT1 genes

Phylogenetic analysis was performed on the exon 1 region of UGT1E genes of chicken, ostrich, turkey, canary, Japanese quail (*Coturuix japonica*), mallard (*Anas platyrhynchos*), bald eagle (*Haliaeetus leucocephalus*), peregrine falcon (*Falco peregrinus*), barn owl (*Tyto alba*), Adelie penguin (*Pygoscelis adeliae*), Northern fulmar (*Fulmarus glacialis*), red-throated loon (*Gavia stellate*), human (*Homo sapiens*) and rat, which were retrieved using the NCBI basic local alignment search tool (BLAST). UGT1E genes used in phylogenetic analysis are shown in Supplementary Table S3. These birds were not the same species, but were closely related to those used in the UGT activity test. Therefore, we could combine the results of UGT activity test and genetic analysis. The deduced amino acid sequences were aligned using multiple sequence comparison by log expectation (MUSCLE) and employed for model selection and construction of maximum likelihood trees (bootstrapping = 500) in Molecular Evolutionary Genetics Analysis X (MEGAX) (Kumar et al., 2018). The best model (Jones, Taylor, and Thorton + G +I model) was used for phylogenetic analysis. All positions with less than 95% site coverage were eliminated, i.e., fewer than 5% alignment gaps, missing data, and ambiguous bases were allowed at any position (partial deletion option). Marbled flounder (*Pleuronectes yokohama*) UGT1B1/1B2 and human UGT2A1/2B4 genes were used as outgroups. This analysis involved 94 amino acid sequences. There were a total of 235 positions in the final dataset.

2.7. Synteny analysis of avian UGT1E genes

NCBI's Genome Data Viewer (https://www.ncbi.nlm.nih.gov/genome/gdv/) was used to visualize chromosomal synteny maps for each avian species used in the phylogenetic analysis. The latest genome assemblies were used: chicken Annotation Release 104, turkey Annotation Release 102, Japanese quail Annotation Release 100, mallard Annotation Release 102, ostrich Annotation Release 100, canary Annotation Release 101, peregrine falcon Annotation Release 101, bald eagle Annotation Release 100, barn owl Annotation Release 100, Adelie penguin Annotation Release 100, Northern fulmar Annotation Release 100, and red-throated loon Annotation Release 100. NCBI BLAST (blastn) of the GenBank database (newest version March 2017) was used for additional confirmation of missing genes (e-value < $3e^{-3}$). Individual 84 UGT protein sequences were used as query sequences. The numbers of functional UGT1E genes and pseudogene were investigated in each avian species.

2.8. Total RNA extraction and cDNA synthesis

We investigated the nine chicken UGT1E isoforms as a model of avian UGT. Total RNA was extracted from the livers of chickens using NucleoSpin® RNA (Takara, Tokyo, Japan). The purity and quantity of RNA were determined by electrophoresis as well as spectrophotometry using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE). The ratio of absorbance at 260 nm and 280 nm (A 260/280) and the ratio of absorbance at 260 nm and 230 nm (A 260/230) were generally ≥ 2. Total RNA (10 μg) was reverse transcribed using ReverTra Ace (Toyobo, Osaka, Japan) according to the manufacturer's instructions.

2.9. Plasmid construction for quantitative real-time PCR

Plasmids were constructed to plot standard curves for calculation of cDNA copy numbers. The cDNA was amplified by PCR using specific primers for nine chicken UGT1E isoforms and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard, as shown in Supplementary Table S4. The nine chicken UGT1E isoforms were submitted to the NCBI database, and named from chicken UGT1E no. 1 to no. 9 according to their locus on the chromosome; UGT1E no. 1 is located closest to exon 2-5 and chicken UGT1E no.9 is farthest from exon 2-5. Correspondences with the names of chicken UGT1Es by the UGT Nomenclature Committee are shown in Supplemental Table S4. PCR was performed using SappireAmp® (Takara) according to the following thermal profile: one cycle of 30 s at 94°C followed by 35 cycles of 5 s at 98°C, 5 s at 60°C, and 5 s at 72°C, with a final extension for 1 minute at 72°C. Plasmids were constructed with the PCR products and pCR2.1-TOPO vector using a TOPO TA Cloning Kit (Thermo Fisher Scientific).

2.10. Quantitative real-time PCR

The mRNA expression levels of chicken UGT1E isoforms in the liver were investigated by qPCR. The gene-specific quantitative real-time PCR primers (Supplementary Table S4) were synthesized by Sigma-Aldrich. The efficiency of all primers was 98%−102%. Quantitative real-time PCR was performed with the StepOne Plus Real-Time system (Thermo Fisher Scientific). The PCR mixtures consisted of Fast SYBR Green Master Mix (Thermo Fisher Scientific), forward and reverse primers (200 nM each), and cDNA derived from RNA in a total volume of 10 μl. All samples 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, melting curve analysis was performed in the range of 60°C−95°C. PCR products were confirmed as single fragments by electrophoresis and direct sequencing. The gene dosages of

the nine UGT1E isoforms were calculated from the Ct values. The gene expression levels were then compensated by gene dosages of GAPDH.

2.11. Statistical analyses

All kinetics parameters, including maximum velocity (V_{max}), the Michaelis–Menten constant (K_m), and the V_{max}/K_m ratio, were determined using the Michaelis–Menten equation and GraphPad Prism version 5.0 for Windows (GraphPad Software, San Diego, CA). Statistical analyses were performed using JMP 11 (SAS Institute Inc, Cary, NC). In quantitative real-time PCR, Student's *t* test and Tukey's test were used for comparing mRNA expression levels between sexes and each UGT1E isoform, respectively. Differences at $p < 0.05$ were considered significant and those at $p < 0.01$ were regarded as highly significant.

3. Results

3.1. UGT activities in avian liver microsomes

In the investigation of reduced CO spectrum of P450 as an indicator of enzymatic condition, a defined peak of P450 at 450 nm could be observed in all microsomal samples. The quality of P450, which is an XME as well as UGT, was conserved. Therefore, we regarded the quality of UGT in microsomes as conserved.

The Michaelis–Menten plots and parameters representing UGT activities in each species are shown in Table 1 and in Figs. 1, 2, and 3. There were large interspecies differences in the glucuronidation activities of β-estradiol, 1-hydroxyperene, and acetaminophen.

With regard to the β-estradiol-3-glucuronidation activities, the V_{max}/K_m values were very low in great horned eagle (= 0.023 nmol/mg/min) and white-tailed eagle (= 0.025 nmol/mg/min), but higher in turkey (= 0.738 nmol/mg/min) and chicken (= 0.333 nmol/mg/min) (Figure 1). In the UGT activity test of 1-hydroxypyrene, lower V_{max}/K_m values were observed in Humboldt penguin (= 0.004 nmol/mg/min) and great horned owl (below the limit of detection), with higher values in turkey (= 0.797 nmol/mg/min), ostrich (= 0.683 nmol/mg/min), and rat (= 0.665 nmol/mg/min) (Figure 2). With regard to acetaminophen glucuronidation, lower V_{max}/K_m values were also observed for the great horned owl (= 0.029 nmol/mg/min) and Humboldt penguin (below the limit of detection), with higher values in rat (= 0.771 nmol/mg/min) and canary (= 0.763 nmol/mg/min) (Figure 3).

3.2. Phylogenetic analysis of avian UGT1E genes in comparison to mammalian UGT1A isoforms

Figure 4 shows a phylogenetic tree of avian UGT1E and mammalian UGT1A isoforms. Avian UGT1E and mammalian UGT1A isoforms formed isolated clusters. Mammalian UGT1A isoforms formed four large clusters, i.e., UGT1A1, UGT1A2 – 1A5, UGT1A6, and UGT1A7 – UGT1A10. In this study, avian UGT1E isoforms were classified into six large groups represented as clades I, II, III, IV, V, and VI in Figure 4.

3.3. Synteny analysis of avian UGT1E isoforms

The locus of avian UGT1E genes in each avian species are shown in Figure 5. All UGT1E isoforms were located between USP40 (ubiquitin specific peptidase 40) and SH3BP4 (SH3 domain binding protein 4) genes in avian species. The numbers of functional UGT1E genes and pseudogenes were counted in each species (Table 2). The locations of Turkey UGT 1E genes (XM_010726367, XM_019610789), Fulmar UGT1E genes (XM_009575112, XM_009583991, XM_009586327), and Adelie penguin UGT 1E gene (XM_009323405) on

the chromosome are unknown because of a lack of gene information.

3.4. mRNA expression levels of different Chicken UGT1E isoforms

The mRNA expression levels of nine chicken UGT1E isoforms were evaluated by quantitative real-time PCR (Figure 6). The mRNA expression level of chicken UGT1E134 (=0.689 in male, 0.323 in female) was higher than those of the other nine isoforms ($p < 0.05$). There were significant differences in expression levels between sexes in chicken UGT1E4 (=0.0482 in male, 0.0118 in female) ($p < 0.05$) and highly significant differences in chicken UGT1E5 (=0.0404 in male, 0.0196 in female), UGT1E6 (=0.0369 in male, 0.0178 in female), UGT1E7 (=0.0391 in male, 0.0186 in female), UGT1E9 (=0.0140 in male, 0.0068 in female), and UGT1E13 ($p < 0.01$).

4. Discussion

4.1. Glucuronidation of β-estradiol among avian species

Clear interspecies differences in β-estradiol-3-glucuronidation were observed in UGT activity test. In contrast, Kakehi et al. (2015) reported only slight interspecies differences in mammalian β-estradiol-3-glucuronidation. β-Estradiol is conjugated by multiple UGT1A isoforms, and UGT1A1 is the most important for β-estradiol-3-glucuronidation in mammals (Hong et al., 2007). Furthermore, bilirubin is metabolized mainly via liver UGT1A1 (Hong et al., 2007), and low UGT1A1 activity leads to death by hyperbilirubinemia in mammals (Iyanagi 1991). However, birds may be able to survive even with very low UGT activity as there is another bilirubin metabolic pathway. These difference in β-estradiol-3-glucuronidation and bilirubin glucuronidation between mammals and birds may be attributable to other metabolizing enzymes involved in this reaction. In heme metabolism of birds, biliverdin is excreted rather than bilirubin, and therefore birds do not need to metabolize bilirubin by glucuronidation (Lin et al., 1974). This difference in metabolic pathway of heme compared with mammals may be one reason why *in vitro* interspecies differences in glucuronidation to β-estradiol were observed for avian species.

4.2. Avian interspecies differences in UGT1-dependent xenobiotics metabolism

In the *in vitro* UGT activity test for all substrates, higher UGT1E activity was observed in herbivorous and omnivorous birds, such as turkey or ostrich, than in carnivorous birds, such as raptors and penguin. Similarly, herbivorous mammals have higher XMEs activities compared to carnivores, as herbivores have to eliminate plant-derived toxins from their diet (Johanna. 2009). As carnivorous birds are top predators of the ecosystem, they are exposed to and accumulate high levels of environmental pollutants (Lourenço et al., 2011). Due to the high accumulation and low metabolic capacity, carnivorous birds seemed to be more sensitive to xenobiotics than herbivorous and omnivorous species.

4.3. Phylogenetic characteristics of avian UGT1Es

In the phylogenetic tree, avian UGT1E and mammalian UGT1A isoforms were located separately, indicating that avian UGT1Es have evolved independently from mammalian UGT1As. In biological classification, birds are more closely related to reptiles than mammals (Mindell et al., 1999), and therefore birds may have UGT isoforms in common with reptile species rather than mammals. This hypothesis is in agreement with the research of Haiyan et al. (2010), who reported that the zebra finch and lizards have UGT isoforms derived from the

same ancestral variable exon, which mammals did not possess.

Previous report suggested that UGT1E genes are classified into 6 groups depends on their evolutionary relationship (Kawai et al., 2018 accepted), although it did not include canary and quail. This study also indicated that UGT1E genes of canary and quail were classified into 6 groups. By combining the results of phylogenetic and synteny analyses, the relationships between gene locus and substrate specificity of avian UGT1E isoforms were estimated (Figure 5). It suggested that the variety of the count of UGT1E_group III implies UGT1E_group III would play major role in metabolizing exogenous compounds in avian species. This was consistent with previous report (Kawai et al., 2018 accepted). In this study, the number of UGT1E_group VI also showed variety especially in Galloanserae species. It suggested that UGT1E_group VI could also contribute to xenobiotic metabolism in Galloanserae species. On the other hand, it is difficult to determine which UGT1E isoforms that metabolize endogenous substrates. In case of mammals almost all species possess UGT1A1 even in carnivorous species (Kakehi et al. 2015). However we could not find no UGT1E group in common in avian species. It seems that many avian species possess UGT1E_group I genes (Kawai et al., 2018 accepted) and play role in metabolizing endogenous compounds, but there are exceptions such as owl, fulmar, Adelie penguin.

4.4. Numbers of functional genes and pseudogenes of avian UGT1Es

Recently we reported that feeding habits affected the number of UGT1E genes in avian species (Kawai et al., 2018 accepted). In this study, the results of synteny analysis and the count of UGT1E genes also supported that feeding habits affect the number of functional and pseudogenes of avian UGT1Es. Carnivorous birds tended to have fewer functional UGT1E genes and some genes had become pseudogenes, compared to herbivorous birds (Figure 5 and Table 2). In the taxonomy of birds, falcons are more closely related to canaries than eagles and owls (Hackett et al., 2008). However, falcons have only three functional UGT1E isoforms and one pseudogene, as in other raptors. This suggested that the numbers of UGT1Es are decreasing by carnivorous feeding. Furthermore, the total numbers of functional UGT1Es in each avian species reflected the interspecific differences of *in vitro* UGT activity. Avian species with low *in vitro* UGT activity, such as eagle, owl, and penguin, had fewer total numbers of functional UGTs and some pseudogenes, while species with high *in vitro* UGT activity, such as turkey, chicken, ostrich, and canary, had many functional UGT1E isoforms. Therefore, the low *in vitro* UGT activity in some avian species could be due to the small number of functional UGT1Es and pseudogenes. This speculation is in agreement with a previous report regarding mammalian UGT1As indicating that cats and pinniped species showed very low UGT activities due to pseudogenes of UGT1A6 or fewer UGT1A isoforms (Kakehi et al., 2015).

UGT families are thought to have undergone birth-and-death evolution, where new genes were

created by gene duplication with some duplicated genes remaining in the genome, whereas others were deleted or became pseudogenes through deleterious mutations (Nei and Rooney, 2005). In mammals, the variable UGT1A exons of the bilirubin and phenol groups appear to have duplicated separately from only two ancestral variable exons (Zhang et al., 2004). In contrast, avian UGT1Es would have evolved from 6 ancestral exons (Kawai et al., 2018 accepted). This evolutionary difference in that avian UGT1Es may have evolved from many ancestral exons suggests that birds have needed to metabolize various exogenous or endogenous compounds from ancestral species.

4.5. Prediction of important chicken UGT1E isoforms

All of the chicken UGT1E isoforms showed sex-related differences, with higher mRNA expression levels in males than in females. In mammalian species, the expression levels of some UGT isoforms are higher in males than in females, but some isoforms are dominantly expressed in females. For example, UGT2B1 is more abundant in male mice, but UGT1A1 and UGT1A5 are expressed at higher levels in females (Buckley et al., 2007). As the mRNA expression levels of chicken UGT1E were higher in males than females, this suggests that the chicken has unique metabolic pathways compared to mammals.

The high mRNA expression levels of chicken UGT1E13 compared to other UGT1E isoforms may indicate a major role of this isoform in metabolism of xenobiotics, such as drugs or environmental pollutants. The most abundant UGT1A isoform in mice is UGT1A1 (Peng et al., 2013), which conjugates endogenous substrates. As mentioned above, however, in chicken that is Galloanserae species, UGT1E13 that belongs UGT1E_group VI seemed to be involved in metabolism of exogenous substrates. It is noteworthy that the UGT1A isoform with highest expression in mammals metabolizes endogenous substrates, while in chicken it may contribute to xenobiotic metabolism. This difference suggests that chicken may have evolved with less use of UGT1Es to metabolize endogenous compounds compared to mammalian species.

However, we should note that this study focused on UGT1E isoforms in chicken only. Further studies are required to investigate the expression levels and substrate specificities of avian UGT1E isoforms belonging to the UGT1E_group III in other avian species. Especially, investigation of UGT1E isoforms in carnivorous birds is needed because they showed very low *in vitro* UGT metabolic ability. Examination of the expression levels of UGT1E isoforms of carnivorous birds, or investigating the substrates specificity of UGT1E isoforms of them may yield insight into why they showed low UGT activities.

5. Conclusion

The results of the present study suggested that carnivorous birds may have lower UGT activities

than herbivorous species. One reason for the lower UGT activities in carnivorous birds is that these species have fewer UGT1E isoforms. We predicted that some UGT1E isoforms would be especially important for exogenous substrate metabolism in chicken. This study will help to understand xenobiotic metabolism and to allow more accurate prediction of the toxic effects of environmental chemicals in avian species and finally lead to improved conservation of wild birds.

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Supplementary data

Supplementary data to this article can be found online at https://.

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679 Figure Legends

680

681 **Figure 1.**

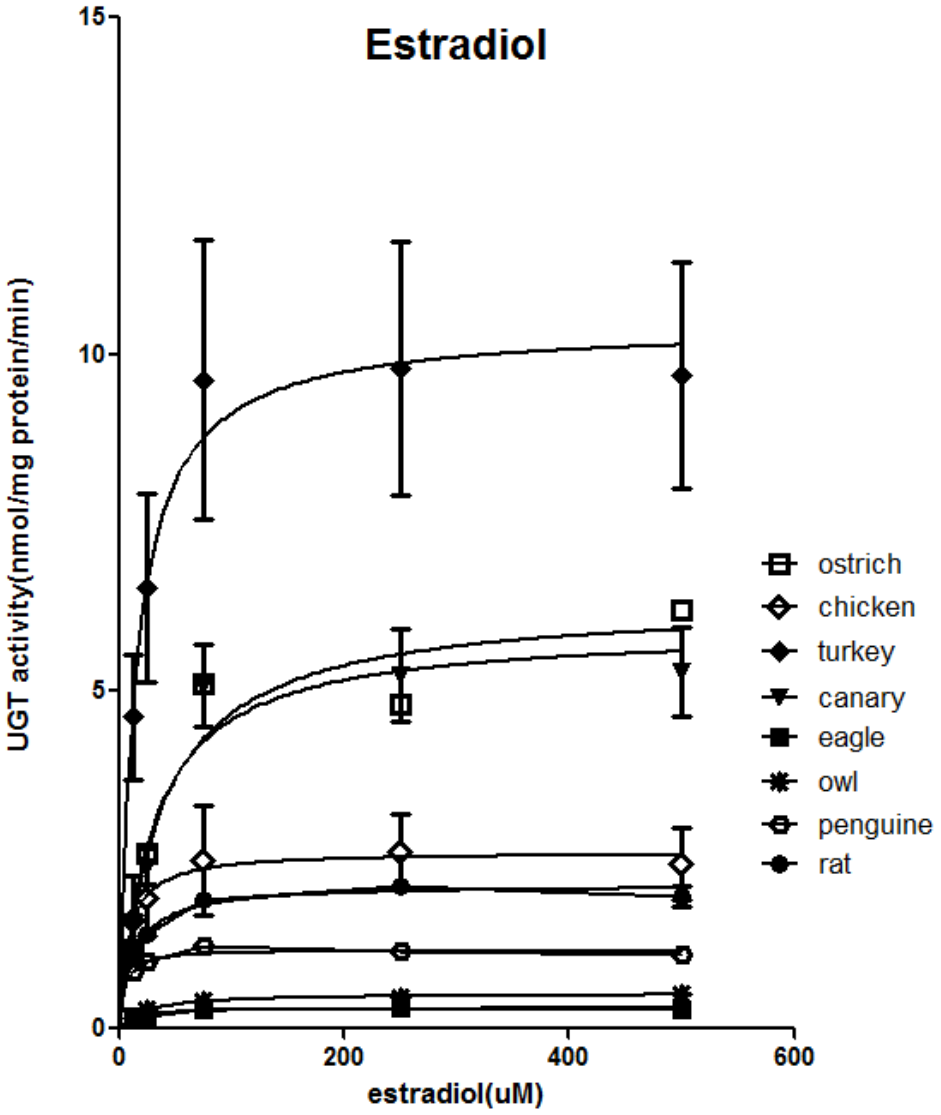

Figure 1. β-Estradiol UGT activity

Enzyme kinetics for the glucuronidation of β-estradiol in chickens, turkeys, ostriches, canaries, white-tailed eagles, great horned owl, Humboldt penguin, and rats. The final β-estradiol concentration was varied between 12.5 µM and 500 µM. Each data point for chicken, turkey, ostrich, canary, and rat represents the mean of three animals and error bars represent the S.D. The numbers in other species were for only one individual and therefore there are no S.D. values.

Figure 2.

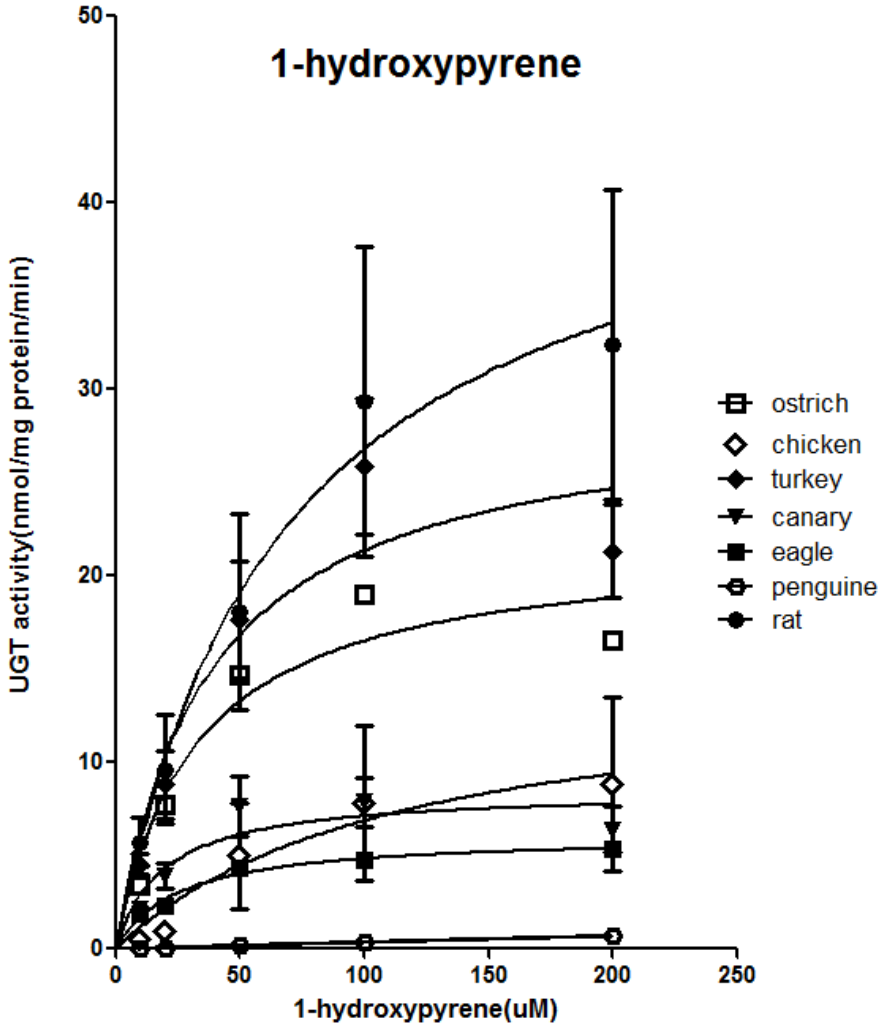

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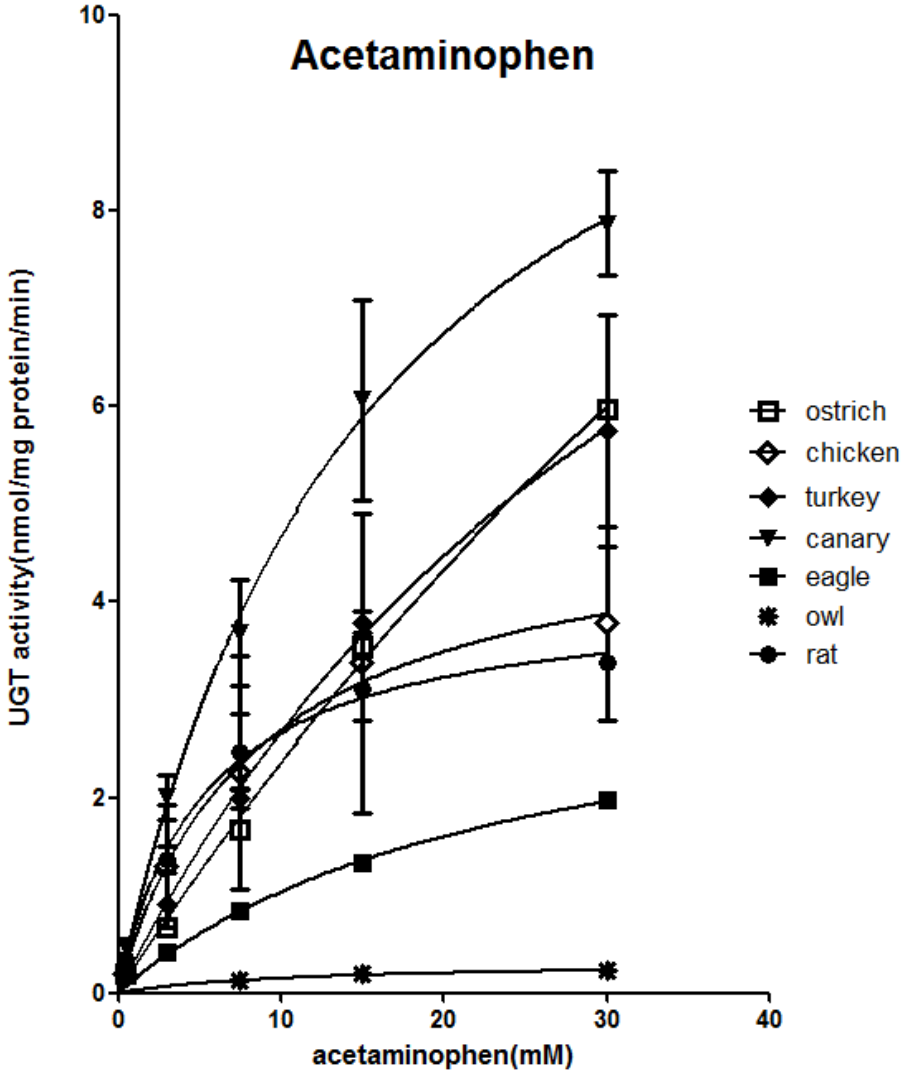
Figure 2. 1-Hydroxypyrene UGT activity

Enzyme kinetics for the glucuronidation of 1-hydroxypyrene in chicken, turkey, ostrich, canary, white-tailed eagle, Humboldt penguin, and rat. The final 1-hydroxypyrene concentration was varied between 10 µM and 200 µM. Each data point for chicken, turkey, ostrich, canary, and rat represents the mean of three animals and error bars represent the S.D. The numbers in other species were for only one individual and therefore there are no S.D. value.

720 **Figure 3.**



745

746 **Figure 3.** Acetaminophen UGT activity

747 Enzyme kinetics for the glucuronidation of acetaminophen in chicken, turkey, ostrich, canary,
748 white-tailed eagle, great horned owl, Humboldt penguin, and rat. The final acetaminophen
749 concentration was varied between 0.5 mM and 30 mM. Each data point for chicken, turkey,
750 ostrich, canary, and rat represents the mean of three animals and error bars represent the S.D.
751 The numbers in other species were for only one individual and therefore there are no S.D.
752 values.

Figure 4. Phylogenetic trees of the UGT isoforms using mammalian UGT1A and avian UGT1E amino acid sequences.

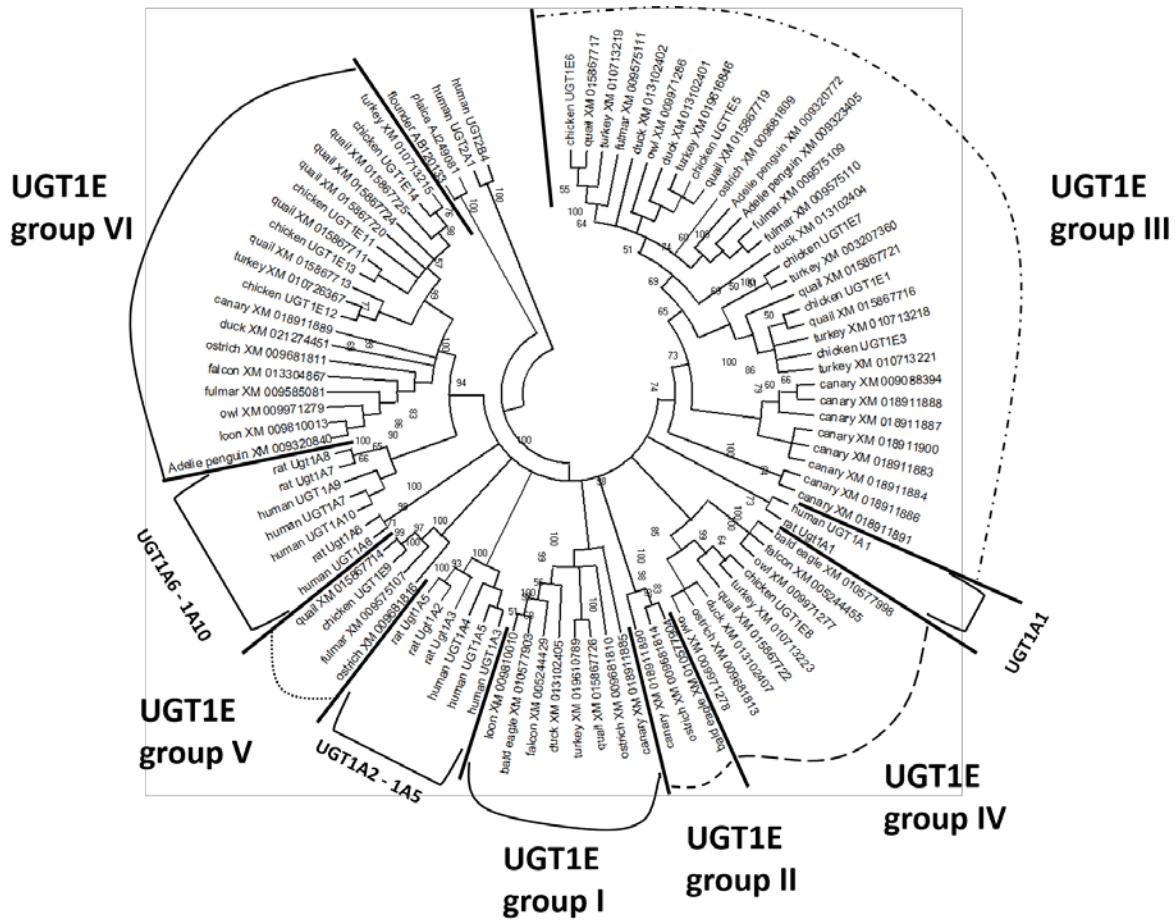
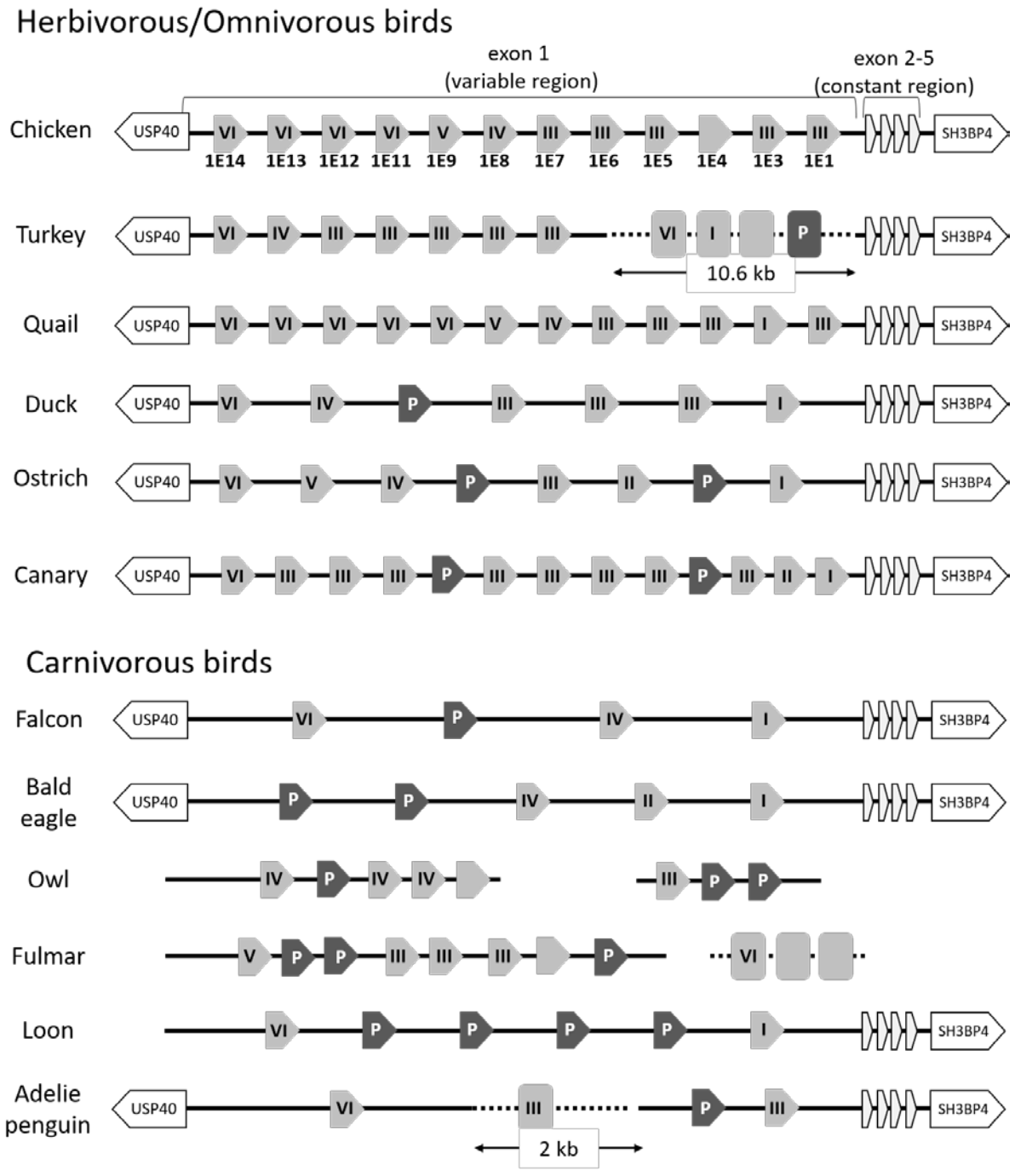


Figure 4. Phylogenetic trees of the UGT isoforms using mammalian UGT1A and avian UGT1E amino acid sequences.

A phylogenetic tree was constructed using avian UGT1E and mammalian UGT1A isoforms. The numbers next to the branches indicate the number of occurrences per 100 bootstrap replicates. Gene names follow the names registered in the NCBI database. The deduced amino acid sequences were aligned on MUSCLE and used for model selection and construction of maximum likelihood trees (bootstrapping = 100) using MEGAX (Kumar et al., 2018). The best model (Jones, Taylor, and Thorton + G + I model) was used for phylogenetic analysis. All positions containing gaps and missing data were eliminated. Marbled flounder (Pleuronectes yokohama) UGT1B1/1B2 and human UGT2A1/2B4 genes were used as outgroups. Mammalian UGT1A isoforms made three clades, UGT1A1, UGT1A2 – UGT1A5, and UGT1A6 – UGT1A10. Avian UGT1E isoforms were divided into 6 clades: UGT1E_group I - VI.

Figure 5.



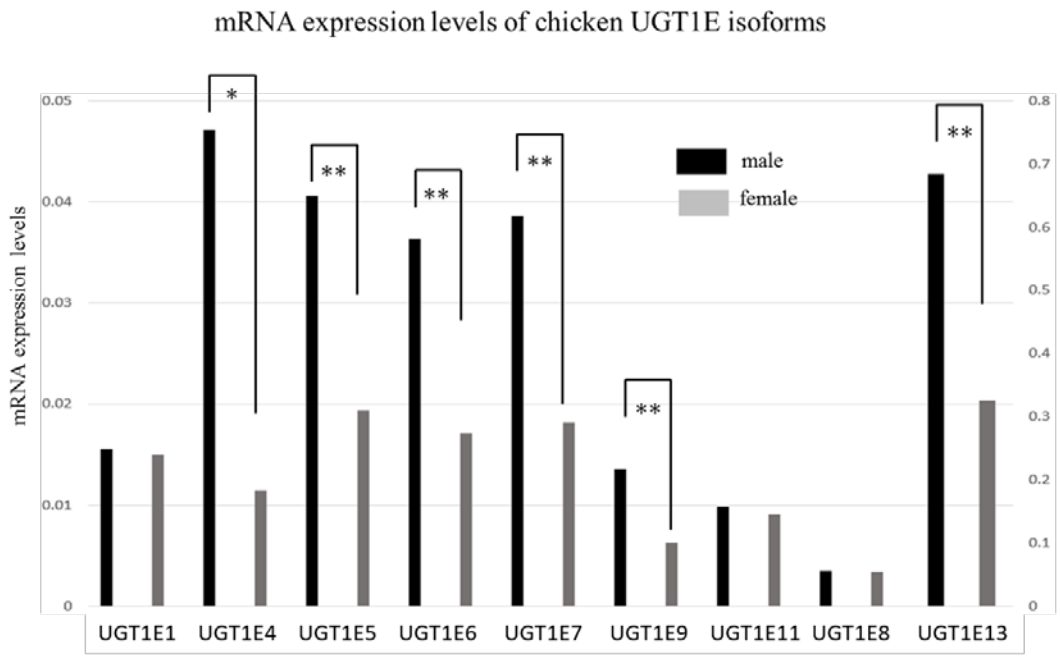
794

795 **Figure 5.** Synteny analysis of avian UGT1Es

796 NCBI's Genome Data Viewer was used to visualize chromosomal synteny maps for each avian
797 species. NCBI BLAST was used for additional confirmation of missing genes and orthologous
798 relationships. The UGT1E locus contains multiple first exons and constant exons 2–5. In all
799 avian species examined, UGT1E isoforms were located between USP 40 and SH3BP4. Pale
800 gray blocks indicate functional UGT1E genes and dark gray blocks with the letter "P" indicate
801 UGT1E pseudogenes. In chicken the gene names determined by UGT nomenclature committee
802 were represented under boxes. Figures in each box indicate the gene names based on

phylogenetic analysis (Figure 4). Dotted lines indicate that parts of gene sequence data are lacking in the NCBI database. The locations of some genes represented by rounded corner squares were unknown.

806 **Figure 6.**



807

808 **Figure 6.** mRNA expression levels of chicken UGT1E genes in the liver

809 The twelve of chicken UGT1E isoforms were predicted to the NCBI database and named to
810 chicken UGT1E1 to chicken UGT1E14 depends on the nomenclature committee. Values shown
811 indicate the mRNA expression level of each isoform normalized by GAPDH. The left vertical
812 axis represents the ratio of chicken UGT1E1, UGT1E4, UGT1E5, UGT1E6, UGT1E7,
813 UGT1E9, UGT1E8, and UGT1E11 mRNA expression levels. The right vertical axis
814 represents the ratio of chicken UGT1E13 mRNA expression level. Each data point represents
815 the mean of three animals. Error bars represent S.D. Blue bars represent results for male
816 chickens, while red bars represent results for female chickens. * Significant differences in
817 mRNA expression levels between male and female chickens ($p < 0.05$). ** Highly significant
818 differences in mRNA expression levels between male and female chickens ($p < 0.01$).

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820

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Table 1: Michaelis-Menten Constants for Glucuronidation

Rat	Turkey	Ostrich	Chicken	Canary	White-tailed eagle	Great horned owl	Humboldt penguin
0.176 ± 0.025	0.738 ± 0.586	0.180 ± 0.032	0.333 ± 0.022	0.203 ± 0.019	0.025 ± 0.008	0.023	0.276
2.131 ± 0.072	10.42 ± 1.128	5.53 ± 0.701	2.617 ± 0.356	5.916 ± 0.492	0.308 ± 0.017	0.524	1.170
12.14 ± 2.272	14.12 ± 7.383	35.12 ± 14.93	7.87 ± 6.57	29.08 ± 9.67	12.51 ± 3.42	23.04	4.246
0.665 ± 0.128	0.797 ± 0.183	0.683 ± 0.064	0.129 ± 0.031	0.445 ± 0.021	0.238 ± 0.019	N.D.	0.004
44.86 ± 12.85	29.17 ± 4.369	21.77 ± 3.376	14.69 ± 10.18	8.463 ± 1.33	6.059 ± 0.362	N.D.	8.861
67.45 ± 47.01	36.62 ± 16.42	3.188 ± 15.48	113.7 ± 18.68	19.00 ± 11.07	25.48 ± 5.130	N.D.	2326
0.771 ± 0.098	0.333 ± 0.138	0.261 ± 0.062	0.595 ± 0.032	0.763 ± 0.182	0.148 ± 0.023	0.029	N.D.
4.086 ± 0.378	13.76 ± 5.846	26.00 ± 7.387	4.951 ± 1.896	12.14 ± 1.960	3.502 ± 0.401	0.318	N.D.
5.299 ± 1.580	41.34 ± 26.81	99.79 ± 35.18	8.315 ± 8.563	15.91 ± 5.394	23.64 ± 4.933	11.08	N.D.

β- estradiol	
*V*max/*K*m (µmol/ min/ ng)	
*V*max (pmol/ min/ ng)	
*K*m (µM)	
1-hydroxy pyrene	
*V*max/*K*m (µmol/ min/ ng)	
*V*max (nmol/ min/ ng)	
*K*m (µM)	
Acetaminophen	
*V*max/*K*m (µmol/ min/ ng)	
*V*max (nmol/ min/ ng)	
*K*m (µM)	

Table 2: Numbers of UGT1E isoforms in each avian species

Species	Functional genes							Pseudogenes
	Total	I	II	III	IV	V	VI	unknown	
Herbivorous/ Omnivorous birds									
Ostrich	6	1	1	1	1	1	1		2
Chicken	12	0	0	5	1	1	4	1	0
Turkey	10	1	0	5			3	1	1
Japanese quail	12	1	0	4	1	1	5		0
Mallard	6	1	0	3	1	0	1		1
Canary	11	1	1	8	0	0	1		2
Carnivorous birds									
Peregrine falcon	3	1	0	0	1	0	1		1
Barn owl	5	0	0	1	3	0	0	1	3
Bald eagle	3	1	1	0	1	0	0		2
Red-throated loon	2	1	0	0	0	0	1		4
Northern fulmar	8	0	0	3	0	1	1	3	3
Adelie penguin	3	0	0	2	0	0	1		1

The numbers of possibly functional genes and pseudogenes of avian UGT1E isoforms were investigated. The numbers of isoforms located in each groups (i.e., I - VI) in the phylogenetic tree (Fig 4.) were counted. Gene information of UGT1E was partly lacking in turkey, red-throated loon, and Northern fulmar, so the minimum expected numbers of UGT1E genes are shown in the table.