Age estimation based on blood DNA methylation levels in brown bears

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Abstract

Age is an essential trait for understanding the ecology and management of wildlife. A conventional method of estimating age in wild animals is counting annuli formed in the cementum of teeth. This method has been used in bears despite some disadvantages, such as high invasiveness and the requirement for experienced observers. In this study, we established a novel age estimation method based on DNA methylation levels using blood collected from 49 brown bears of known ages living in both captivity and the wild. We performed bisulfite pyrosequencing and obtained methylation levels at 39 cytosine-phosphate-guanine (CpG) sites adjacent to 12 genes. The methylation levels of CpGs adjacent to four genes showed a significant correlation with age. The best model was based on DNA methylation levels at just four CpG sites adjacent to a single gene, SLC12A5, and it had high accuracy with a mean absolute error of 1.3 years and median absolute error of 1.0 year after leave-one-out cross-validation. This model represents the first epigenetic method of age estimation in brown bears, which provides benefits over tooth-based methods, including high accuracy, less invasiveness, and a simple procedure. Our model has the potential for application to other bear species, which will greatly improve ecological research, conservation, and management.

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Running title: An epigenetic clock in brown bears

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Abstract

Age is an essential trait for understanding the ecology and management of wildlife. A conventional method of estimating age in wild animals is counting annuli formed in the cementum of teeth. This method has been used in bears despite some disadvantages, such as high invasiveness and the requirement for experienced observers. In this study, we established a novel age estimation method based on DNA methylation levels using blood collected from 49 brown bears of known ages living in both captivity and the wild. We performed bisulfite pyrosequencing and obtained methylation levels at 39 cytosine-phosphate-guanine (CpG) sites adjacent to 12 genes. The methylation levels of CpGs adjacent to four genes showed a significant correlation with age. The best model was based on DNA methylation levels at just four CpG sites adjacent to a single gene, *SLC12A5* , and it had high accuracy with a mean absolute error of 1.3 years and median absolute error of 1.0 year after leave-one-out cross-validation. This model represents the first epigenetic method of age estimation in brown bears, which provides benefits over tooth-based methods, including high accuracy, less invasiveness, and a simple procedure. Our model has the potential for application to other bear species, which will greatly improve ecological research, conservation, and management.

Key words

aging; DNA methylation; epigenetic clock; age estimation; brown bear; wildlife management

Introduction

Age is an important factor in the study of wildlife ecology. Age information is essential to establishing life history characteristics such as growth rate, age of maturity, and age of death (Morris 1972). In addition, survival and fecundity rates are inextricably linked to age; as animals age, they reach actuarial (i.e., decreasing survival probability with age) and reproductive senescence (Williams 1957; Monaghan et al. 2008; Nussey et al. 2013; Gaillard & Lemaître 2017; Gaillard & Lemaître 2020). These factors are closely related to population dynamics (Oli & Armitage 2004) and in turn to the conservation and management of animal species (Robert et al. 2015; Colchero et al. 2019; Tidière et al. 2021).

Consequently, biologists must determine the ages of animals, although estimating age based on appearance is difficult for many species. Therefore, the otoliths and scales of fish (Kimura et al. 1979) and the wax plugs of baleen whales (Purves 1955) have been used for age estimation. In other cases, teeth have been used to assess age in various wildlife species. The method of counting the annual rings of tooth cementum has been used with pinnipeds (Scheffer 1950; Laws 1952; Scheffer & Myrick 1980), and subsequently, the number of laminations in teeth was employed to estimate age in toothed whales (Nishiwaki et al. 1958). In addition, cementum annuli have been widely adopted to determine the age of terrestrial mammals (Thomas 1977). However, some difficulties face age estimation using cementum annuli. First, study animals must be captured to remove a tooth, which limits the target to dead or anesthetized individuals. Moreover, pulling teeth from living animals is highly invasive. Second, accuracy may differ between skilled and less-experienced workers due to the precision required for this technique. Third, cementum annuli become more difficult to read in older individuals. Fourth, cementum annuli are thought to form at different rates depending on climate and nutritional stress, and these informations are not always available for target species or regions (Rolandsen et al. 2008).

Brown bears (*Ursus arctos*) live for 20–30 years (Interagency Grizzly Bear Committee 1987). Pregnant female bears give birth from late January to early February (Friebe et al. 2014) during winter hibernation, which lasts for 3–7 months (González-Bernardo et al. 2020). Offspring become independent from their mothers at 1.5 or 2.5 years of age (Shimozuru et al 2017). The minimum age at first parturition was 4 years (Mano & Tsubota 2002), and physical growth terminated around 5 and 8 years of age for females and males,

respectively (Shirane et al. 2020). Individual and seasonal variations in body size make identification of bear age by appearance almost impossible (Shirane et al. 2020; Shirane et al. 2021) except for cubs-of-the-year, and therefore tooth-based age estimation has been used to determine bear ages. In Europe and the United States, brown bears have been protected or carefully managed after dramatic population decreases (Zedrosser et al. 2001; Mattson & Merrill 2002). On the other hand, conflicts such as crop depredation, intrusion into human residential areas, and attacks on livestock and humans have become serious problems, and management agencies have developed policies to reduce these conflicts (Can et al. 2014; Bombieri et al. 2019). Controlling bear populations via legal hunting and culling is one such policy. Bears are vulnerable to over-harvesting due to their low reproductive rate, and reduced populations require many years to recover (Miller 1990). Therefore, knowledge of the age structure is crucial to understanding their ecology, as well as to the development of appropriate strategies for conservation and management of bears.

Recently, as an alternative method for age estimation, DNA methylation levels have been employed as an indicator (Bocklandt et al. 2011; Koch & Wagner 2011). DNA methylation is an epigenetic mechanism involving the transfer of a methyl group onto the C5 position of cytosine to form 5-methylcytosine, which occurs predominantly on cytosines located within cytosine–guanine dinucleotide (cytosine-phosphate-guanine; CpG) sites in vertebrates (Bogdanović & Veenstra 2009). DNA methylation regulates gene expression by inhibiting the binding of transcriptional activators to DNA or altering chromatin states to inhibit transcription factor binding (Moore et al. 2013; Rose & Klose 2014). In addition to the relationship between DNA methylation and gene expression, research has demonstrated that the degree of DNA methylation changes with age (Jones et al. 2015), providing a foothold for its application to age estimation (De Paoli-Iseppi et al. 2017). In early epigenetic research efforts for age estimation, the focus was on forensic research in humans using various biological samples, including blood, muscle, saliva, buccal swabs, and semen (Horvath 2013; Lee et al. 2015; Bekaert et al. 2015). Subsequently, similar techniques have been established for laboratory animals such as mice (Wang et al., 2017; Petkovich et al., 2017; Stubbs et al., 2017) and naked mole rats (*Heterocephalus glaber* ; Lowe et al. 2020), companion animals such as dogs and cats (Thompson et al. 2017; Qi et al. 2021; Raj et al. 2021), and wild animals such as humpback whales (*Megaptera novaeangliae* ; Polanowski et al. 2014), bottlenose dolphins (*Tursiops truncatus* ; Beal et al. 2019), long-lived seabirds (*Ardenna tenuirostris* ; De Paoli-Iseppi et al. 2019), green turtles (*Chelonia mydas* ; Mayne et al. 2022), chimpanzees (*Pan troglodytes* ; Ito et al. 2018), wolves (*Canis lupus* ; Thompson et al. 2017), and roe deer (*Capreolus capreolus* ; Lemaître et al. 2022). Moreover, body condition and life history factors such as obesity, social status, and hibernation are reportedly associated with the DNA methylation level (Biggar & Storey 2014; Alvarado et al. 2015; Yamazaki et al. 2021).

The main purpose of this study was to establish a novel age estimation method for brown bears based on methylation levels in blood-derived DNA collected from captive and wild bears. Differences in sex and growth environment, including diet, frequency of interactions with conspecifics, hibernation status, and risk of exposure to pathogens, may contribute to epigenetic aging. Therefore, we assessed such influences on epigenetic aging in bears.

Materials and Methods

Ethical Statement

All procedures involved in sample collection from live animals were conducted in accordance with the Guidelines for Animal Care and Use, Hokkaido University, and were approved by the Animal Care and Use Committee of the Graduate School of Veterinary Medicine, Hokkaido University (Permit Number: 1106, 1151, 1152, 15009, 17005, 18-0083, 19-0021, and 20-0146).

Study area, animals, and blood sampling

Captive bears

Blood samples were obtained from 34 brown bears (17 males and 17 females) kept at Noboribetsu Bear Park (Noboribetsu, Hokkaido, Japan; Figure 1) during 2020–2022. Their ages ranged from 2 to 34 years old

(Supplementary Table S_M1). Four individuals were rescued in the wild at 0 years of age and were moved to the facility, while all others were born in the facility. All individuals were fed bear pellets (ZOOFOOD bear; Nosan Co., Kanagawa, Japan), concentrated feed formulated for cows (Soyokazenokaori MG; Nippon Formula Food Manufacturing Co., Ltd., Kanagawa, Japan), dog food (Maibitto; PETLINE Inc., Gifu, Japan), vegetables, and fruits once or twice daily. Water was provided *ad libitum* . Bears fed in winter and did not hibernate. Prior to blood sampling, bears were anesthetized via intramuscular administration of xylazine HCl (1 mg/kg; Selactar; Bayer, Tokyo, Japan) and a 1:1 mixture of zolazepam HCl and tiletamine HCl (2.0–4.0 mg/kg; Zoletil 100; Virbac, Carros, France) using a blow dart. After blood sampling was completed, atipamezole HCl (1 mg/kg; Atipame; Kyoritsu Co., Ltd., Tokyo, Japan) was injected intramuscularly to aid recovery from anesthesia. Blood samples were collected via the medial saphenous vein into vacuum tubes containing ethylenediaminetetraacetic acid disodium (EDTA-2Na) as an anticoagulant. The collected blood samples were stored at -80degC as whole blood or buffy coat until genomic DNA extraction. Buffy coat samples were obtained by centrifuging blood samples at 1,880 x g for 10 minutes.

Wild bears

We used 31 blood samples from 13 wild brown bears (1 male and 12 females) living in the Rusha area (44deg11'N, 145deg11'E) of the Shiretoko Peninsula, eastern Hokkaido, Japan (Figure 1). Seven bears were sampled multiple times at different ages. Their ages ranged from 1 to 26 years old (Supplementary Table S_M1). In this area, long-term bear monitoring surveys have been conducted since 1997 until the present, enabling age determination based on visual and DNA-based identification (Shimozuru et al. 2017).

Bears were anesthetized via intramuscular administration of a 1:1 mixture of zolazepam HCl and tiletamine HCl (5.5 mg/kg; Zoletil 100; Virbac) and 75 μg/kg medetomidine HCL (Dorbene Vet; Kyoritsu Co., Ltd.) based on estimated body weight using an air rifle. After immobilization, bears were weighed, and blood was collected. When blood sampling was completed, atipamezole HCl (375 μg/kg; Atipame; Kyoritsu Co., Ltd.) was injected intramuscularly to aid recovery from anesthesia. Blood samples were collected via the jugular vein into vacuum tubes containing EDTA-2Na as an anticoagulant. The collected blood samples were stored at -80°C as whole blood or buffy coat until genomic DNA extraction. Buffy coat samples were obtained by centrifuging blood samples at 1,880 $\times$ g for 10 minutes.

Additionally, two female cubs-of-the-year that were captured with their mother in a barrel trap were sampled in Shibetsu Town, located in the southeastern part of the Shiretoko Peninsula. Bears were anesthetized using a blow dart for intramuscular administration of a 1:1 mixture of zolazepam HCl and tiletamine HCl (3 mg/kg; Zoletil 100; Virbac) and 40 μg/kg medetomidine HCL (Dorbene Vet; Kyoritsu Co., Ltd.), and then were awakened using atipamezole HCl (200 μg/kg; Atipame; Kyoritsu Co., Ltd.). All other procedures were conducted as described above.

Information about the samples collected for this study is summarized in Supplementary Table S_M1. Ages were determined at the time of blood sampling based on the assumption that all bears were born on February 1 (Friebe et al. 2014).

Genomic DNA extraction and bisulfite conversion

Genomic DNA was extracted from 100 μL EDTA-2Na-treated blood using the DNeasy Blood & Tissue Kit (Qiagen Inc., Tokyo, Japan). Extraction was performed according to the manufacturer's protocol. To adjust for the genomic DNA concentration, the elution volume was 100 μL for whole blood and 150 μL for buffy coat. The concentration of extracted DNA was measured using the NanoDrop 2000c spectrophotometer (Thermo Scientific, Tokyo, Japan). Extracted genomic DNA was stored at -30°C, then bisulfite converted using the EZ DNA Methylation-Gold Kit (Zymo Research, Irvine, CA, USA) according to the manufacturer's instructions, and finally adjusted to 5 ng/μL.

Selection of target genomic locations

We selected target genomic locations adjacent to 12 genes, namely *GSE1* , *VGF* , *SLC12A5* , *SCGN* , *KCNK12* ,*OTUD7A* , *BCL6B* , *POU4F2* , *ELOVL2* , *RALYL* ,*KISS1R* , and *CAPS2* (Table 1). The

methylation levels of CpG sites adjacent to these genes change with age in humans (Day et al. 2013; Florath et al. 2014; Bekaert et al. 2015; Lowe et al. 2018), dogs (Ito et al. 2017; Yamazaki et al. 2021), and cats (Qi et al. 2021). We identified homologous sequences in the genomic regions of brown bears containing the target CpG sites using the Basic Local Alignment Search Tool (BLAST) provided by the National Center for Biotechnology Information.

Polymerase chain reaction and pyrosequencing

Polymerase chain reaction (PCR) and subsequent pyrosequencing were conducted following previously reported methods (Yamazaki et al. 2021). PCR was performed using the TaKaRa EpiTaq HS (for bisulfite-treated DNA) (Takara Bio Inc., Shiga, Japan). PCR and pyrosequencing primers, listed in Supplementary Table S_M2, were designed using Methyl Primer Express v1.0 (Thermo Fisher Scientific, San Jose, CA, USA) and PyroMark Assay Design v2.0.2.5 (Qiagen Inc.). PCR was conducted in two steps so that biotin-modified primers could be used in any region (Yamazaki et al. 2021).

The first PCR step was performed in a total volume of 15 μL containing 0.75 μL of the genomic DNA sample (diluted to contain 3.75 ng DNA), 0.075 μL TaKaRa EpiTaq HS, 1.5 μL 10× EpiTaq PCR Buffer (Mg^{2+} free), 1.5 μL $MgCl_2$, 1.8 μL deoxynucleoside triphosphate (dNTP) mixture, 0.3 μL each of the forward and reverse primers (10 μmol/L), and 8.775 μL molecular-grade water (Nippon Gene, Tokyo, Japan). The PCR conditions were 98°C for 1 min, followed by 35 cycles of 98°C for 10 s, annealing temperature (listed in Supplementary Table S_M2) for 30 s, and 72°C for 30 s. Each sample was run in duplicate. The second PCR step was performed in a total volume of 15.13 μL containing 0.1 μL of the first PCR product, 0.075 μL TaKaRa EpiTaq HS, 1.5 μL 10× EpiTaq PCR Buffer (Mg^{2+} free), 1.5 μL $MgCl_2$, 1.8 μL dNTP mixture, 0.3 μL of the forward primer (10 μmol/L), 0.06 μL of the reverse primer (10 μmol/L), 0.27 μL of the biotin-modified primers (10 μmol/L), and 9.525 μL molecular-grade water (Nippon Gene). The PCR conditions were 98°C for 1 min, followed by 35 cycles of 98°C for 10 s, annealing temperature (listed in Supplementary Table S_M2) for 30 s, and 72°C for 30 s. The success of the first and second PCR amplifications was confirmed by electrophoresis on a 2% agarose gel.

To determine the methylation levels of the target CpGs, pyrosequencing was performed using PyroMark Q48 software (Qiagen Inc., Tokyo, Japan) with PyroMark Q48 Advanced Reagents (Qiagen Inc.) according to the manufacturer's instructions. As PCR for each sample was conducted in duplicate, the average value was taken as the methylation level for that sample. Initially, as a screening step, each target CpG site was analyzed across nine samples to identify age-related genomic locations, i.e., those showing a correlation between age and DNA methylation levels. These samples were selected from captive bears, with the numbers of individuals balanced according to age and sex. From 12 candidate genomic locations, locations with R^2 values greater than 0.8 and a range of methylation level changes greater than 20% were selected for further analysis. Finally, we analyzed the remaining blood samples based on the selected genomic locations and calculated Pearson's product-moment correlation coefficients and p values.

Age estimation model and model validation

To establish an age estimation model, we used only one sample per individual wild bear (i.e., 15 samples from wild bears were used in total). For wild bears sampled multiple times, samples were selected to include as wide an age range as possible, and some were selected randomly (see Supplementary Figure S_M1).

Based on the pyrosequencing results, we generated three age estimation models, including a single regression that requires only the methylation level of one CpG and two multiple regression models (elastic net

regression and a support vector regression [SVR]) that require multiple CpG methylation levels. Age and DNA methylation levels were standardized prior to integration into the model. Single regression models were generated using the R command "lm". Elastic net regression models, a type of penalized regression that has often been used in age estimation models for other species (Horvath et al. 2013), were generated using the R package "glmnet". Optimized parameters (alpha and lambda) were obtained using "cv.glmnet". The SVR models, which are considered better for age estimation than are elastic net regression models (Xu et a. 2015;

Fan et al. 2022), were generated using the R package "e1071". The parameters (cost, gamma, and epsilon) were determined using the "tune" command with fixed settings "type = eps-regression, kernel = radial". We performed leave-one-out cross-validation (LOOCV) to validate all models. LOOCV is a cross-validation method in which only a single dataset is extracted for testing, and all other data are used as training data, which are repeated as many times as the number of samples.

To evaluate whether age, sex, or growth environment affects the deviation of the age estimation model, linear regressions were generated using Δage (predicted age - chronological age) and |Δage| (absolute difference between predicted age and chronological age) as dependent variables and three factors, as well as the interactions among each factor pair, as explanatory variables (Qi et al. 2021). Model construction and selection were conducted using the "lm" command and "MnMIn" package in the R software.

Results

Selection of age-related genomic locations

Through the screening of nine samples, four DNA regions that met the requirements were selected from the 12 candidate genomic locations, including the regions adjacent to *SLC12A5* , *POU4F2* ,*VGF* , and *SCGN* . All results are shown in Supplementary Figures S_R1_1–11.

Correlation between DNA methylation level and chronological age

For each location, multiple CpGs showed significant correlations between methylation level and age (4, 4, 3, and 2 CpGs for *SLC12A5* ,*POU4F2* , *VGF* , and *SCGN* , respectively; Supplementary Figures S_R2_1, 2, 3, and 4). The CpGs that showed the strongest correlations with age for each location are shown in Figure 2 (SLC12A5: correlation coefficient [cor] = 0.98, $p < 0.001$; POU4F2: cor = 0.94, $p < 0.001$; VGF: cor = 0.90, $p < 0.001$; SCGN: cor = 0.90, $p < 0.001$). All results, including the methylation levels of the remaining CpGs, are shown in Supplementary Figures S_R3_1, 2, 3, and 4.

Within-individual change

Wild female bears that were sampled multiple times showed similar trends in methylation level changes with age (Figure 3). Corresponding graphs for the remaining CpG sites are shown in Supplementary Figures S_R4_1, 2, 3, and 4.

Age estimation model

Comparison of models

We constructed three types of age estimation models based on the DNA methylation levels of CpGs adjacent to *SLC12A5* (SLC12A5-1, -2, -3, and -4), *POU4F2* (POU4F2-1, -2, -3, and -4), *VGF*(VGF-1, -2, and -3), and *SCGN* (SCGN-1 and -2) and compared their performances (Table 2; Supplementary Figures S_R2_1, 2, 3, and 4).

The single regression model using the methylation level of SLC12A5-4 showed the best performance; the mean absolute error (MAE) after LOOCV was 1.6 (Figure 4a). The formula for age estimation was as follows:

"Estimated Age" = (-1.962e-11 + 0.9808 × "methylation level of SLC12A5-4") × 10.113 (standard deviation of training data) + 12.550 (mean of training data)

The elastic net regression model selected as the best performing model included the methylation levels of SLC12A5-4, POU4F2-2, and VGF-2; the MAE after LOOCV was 1.5 (Figure 4b). The formula for age estimation is as follows:

"Estimated Age" = (-1.717e-11 + 0.6728 x "methylation level of SLC12A5-4" + 0.1652 x "methylation level of POU4F2-2" + 0.1535 x "methylation level of VGF-2") x 10.113 + 12.550

The SVR model that showed the best performance used the methylation levels of SLC12A5-1, -2, -3 and -4; the MAE after LOOCV was 1.3 (Figure 4c). The R script used to estimate age is available in Supplementary File. Details of the parameters used in the elastic net regression and SVR models are shown in Supplementary Table S_R1.

Influences of age, sex, and growth environment on the model

We used linear regression analysis to identify the factors that affect Δage and |Δage| in the best model (i.e., the SVR model with four CpGs adjacent to *SLC12A5*). When Δage was used as the dependent variable, the best regression model included age, growth environment, and the interaction between age and growth environment as explanatory variables (adjusted $R^2 = 0.1869$) (Table 3 and Figure 5). Among those variables, the interaction between age and growth environment was statistically significant (Figure 5b). When |Δage| was used as the dependent variable, the best regression model included age, growth environment, and the interaction between age and growth environment as explanatory variables (adjusted $R^2 = 0.186$) (Table 4 and Figure 6). Among them, growth environment was statistically significant (Figure 6d). The explanatory variables that were statistically significant for other models (i.e., the single regression model and elastic net regression model) were shown in supplementary tables (Supplementary Tables S_R2, 3, 4, and 5).

Discussion

To the best of our knowledge, this is the first study to establish a method for epigenetic age estimation in bears. We constructed a single regression model using one CpG, designated "SLC12A5-4", which showed the strongest correlation between age and methylation level. Additionally, we constructed six elastic net regression models and six SVR models using various combinations of 13 CpGs. Evaluation based on the MAE of LOOCV suggested that the best SVR model is the model using four CpGs, designated "SLC12A5-1, -2, -3, and -4". This model had the smallest median absolute error (MedAE) and the second smallest root mean square error (RMSE) for LOOCV. The four CpGs included in this model can be covered by a single PCR amplification, suggesting that this model provides the greatest benefits in terms of both accuracy and cost-effectiveness among all tested models. Although the best model in this study targeted only four CpG sites, the accuracy of the model was comparable with that of models developed previously using genome-wide approaches (targeting more than 30 CpG sites) in other carnivore species with shorter life spans than bears. For example, the MedAE values were approximately 0.8 years in studies of dogs, wolves (Thompson et al. 2017), and cats (Raj et al. 2021), whose life spans are approximately one-half to two-thirds of the brown bear lifespan. Furthermore, the DNA methylation levels in seven individuals that were sampled multiple times showed similar increasing trends to those of other samples included in the model. These repeated measurements were all obtained from wild individuals, and the data are valuable because changes in DNA methylation levels were tracked in the wild under highly variable environmental conditions. As shown in Table 1, the MAE, MedAE, and RMSE values were small even when age estimation was performed for wild samples that were not included in the model, indicating that the models we constructed were highly accurate.

The current method is superior to other commonly used age estimation methods in bear species in the following respects. First, this method provides accurate and precise age estimates. Traditionally, tooth-based age estimation has been commonly used for bears (Mundy & Fuller 1964; Marks & Erickson 1966; Stoneberg & Jonkel 1966). Ages estimated using this method are 80–90% consistent with the actual age of the animal. However, if the observer is inexperienced, the accuracy and precision of age estimation are reduced (Mclaughlin et al. 1990). In addition, the error is greater for older than younger bears (Mclaughlin et al. 1990; Harshyne et al. 1998; Costello et al. 2004), as the annuli of older bears are less distinct and interpretable. The current method based on pyrosequencing can avoid such human errors and overcome the technical difficulties facing age estimation. Second, this method is less costly in terms of time, money, and human resources compared with traditional tooth-based age estimation, which requires multiple steps, including decalcification, neutralization, section preparation, staining, and counting of cementum annuli (Tochigi et al. 2018). Depending on the protocol, these steps may take several days (Matson et al. 1993). Third, the current method requires only 100 μL blood, which is much less invasive to the animal than removal of a tooth. Additionally, blood sampling is much easier than tooth removal, as pulling the tooth without

breaking the root requires skill (Costello et al. 2004).

In the best model identified in this study, sex had no effect on Δage (predicted age - chronological age) or |Δage| (absolute difference between predicted age and chronological age). This finding indicates that no sex difference existed in age-dependent DNA methylation changes or individual variability and further suggests that our method can be applied regardless of sex. Among humans, DNA methylation levels change faster in males than in females (Nussey et al. 2013). Adult males have shorter lifespans than those of adult females in many long-lived vertebrates (Clutton-Brock & Isvaran 2007), although it remains unclear whether the shorter lifespans of males are associated with faster epigenetic aging (Hägg & Jylhävä. 2021). Notably, we had small sample sizes for males, especially for wild bears. In contrast to females, which are philopatric, males born in our study region leave the area at the age of 2–3 years (i.e., dispersal behavior (Blanchard & Knight 1991; Shirane et al. 2019)), hindering sample collection from adult males of known ages. In addition, only three captive males over 10 years of age were included in the analysis. Therefore, further study is needed to determine the influence of sex on DNA methylation.

The error represented by |Δage| was larger for wild bears than for captive bears (Table 1), indicating that individual differences in DNA methylation are greater in wild than captive bears. One possible reason for this difference is that captive bears are fed the same type and quantity of food throughout the year in a stable environment, whereas wild bears consume a variety of foods in differing quantities depending on the season (Naves et al. 2006; Shirane et al. 2021). Additionally, foraging strategies differ among individuals even within a population (Servheen & Gunther 2022; Jimbo et al. 2022). Factors linked to lifestyle, including obesity, weight reduction, and overfeeding, have been suggested to affect DNA methylation (Samblas et al. 2019; Yamazaki et al. 2021). Wild brown bears show cyclical annual body mass patterns, with a continuous decrease from the beginning of winter hibernation to summer, and a rapid increase during autumnal hyperphagia (McLellan 2011; Schwartz et al. 2014). In addition, the annual fluctuation in food availability affects body condition (Shirane et al. 2021). The dietary diversity of wild bears and annual fluctuations in food availability may cause greater individual differences in DNA methylation levels compared with captive bears.

Interestingly, Δage tended to decrease with age in wild individuals. This result indicates that older wild individuals have lower age estimates and contradicts the fact that captive animals generally live longer than wild animals (Müller et al. 2010; Lemaître et al. 2013). Animals with short lifespans and high reproductive rates are reported to live longer in captivity than in the wild, but this trend is not always true for species with long lifespans and low reproductive rates (Tidière et al. 2016). Brown bears have a long lifespan and low reproductive rate, which may explain the current results. Another possible explanation is the presence or absence of hibernation periods. Wild brown bears spend 3–7 months hibernating in a reduced metabolic state (González-Bernardo et al. 2020), whereas captive bears in Noboribetsu Bear Park are fed throughout winter and do not hibernate. In general, hibernators live longer than similar-sized non-hibernators (Wilkinson & South 2002; Turbill et al. 2011; Wilkinson and Adams 2019). Recent studies of yellow-bellied marmots, *Marmota flaviventris* , (Pinho et al. 2022) and big brown bats, *Eptesicus fuscu* s, (Sullivan et al. 2022) suggest that this difference arises in part because hibernation slows epigenetic aging. Similarly, in bears, the low metabolic state characteristic of hibernation (Tøien et al. 2011) may reduce the rate of DNA methylation changes, lowering the epigenetic ages of aged individuals. However, this study included a limited number of wild bears, especially aged bears. Further study is needed to clarify this issue.

Among the four genes located adjacent to the CpGs whose methylation levels showed significant correlations with age in bears, *SLC12A5*and *POU4F2* have been reported to show similar correlations in other carnivores (Ito et al. 2017; Raj et al. 2021). *VGF* and*SCGN* show changes in expression during aging at the protein level in T lymphocytes (Busse et al., 2014) and at the mRNA level in blood mononuclear cells (Tan et al. 2012) in humans, although whether those changes are due to changes in the DNA methylation level remains unknown. The gene *SLC12A5* , located adjacent to the CpG site whose methylation levels were used for the best age estimation model, encodes an integral membrane KCl co-transporter that regulates cell volume, net trans-epithelial salt movement, and maintenance of a low intracellular Cl^- concentration in neurons (Payne

et al. 1996). As far as we know, age-related changes in expression at the mRNA or protein levels have never been reported. In humans, the DNA methylation level of the CpG site proximal to this gene is a frequently used target for age estimation in a variety of samples, including blood, saliva, buccal swabs, and hair (Florath et al. 2014; Hong et al. 2017; Hao et al. 2021; Schwender et al. 2021). This finding suggests that age-dependent methylation changes at CpG sites adjacent to *SLC12A5* occur in a tissue-independent manner. In addition, CpGs located in the promoter region of *SLC12A5* showed strong age-related methylation changes in cats (Raj et al. 2021). Although no positive relationship between age and the methylation level of this site was found in dogs (Ito et al. 2017), the present findings suggest that CpG sites adjacent to*SLC12A5* are a useful methylation marker for age estimation in other mammals.

In conclusion, we established an epigenetic age estimation model for brown bears using SVR models and obtained an MAE value of 1.3 years. This value is comparable with those from models established for other animals. The current method is more accurate, easier to perform, and less invasive than conventional tooth-based methods. Notably, our long-term field study enabled the establishment of age estimation models for bears, including both captive and wild bears. Furthermore, this age estimation model may be applicable to other bear species, such as Asian black bears (*Ursus thibetanus*), American black bears (*Ursus americanus*), and polar bears (*Ursus maritimus*). The current study will contribute to ecological research, conservation, and management of bear species.

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Data accessibility

The data obtained from pyrosequencing analyses will be made available in Dryad.

Conflict of interests

The authors have no conflicts of interest to declare.

Author contributions

H.I. to and M.S. (Michito Shimozuru) conceived of the study. N.M., M.Y., M.N., Y.Y., T.T., M.S. (Michito Shimozuru) and S.N. were involved in sample collection. J.Y., H.I., M.I.-M., and H.Q. supported with the technical aspects of the experiment. S.N. performed all laboratory work and constructed each age estimation model. S.N. and M.S. (Michito Shimozuru) wrote the article with inputs from J.Y., T.T., M.S. (Mariko Sashika), H.I. and M.I.-M. All authors reviewed the article.

Tables and figures

Table 1. Target genomic locations and adjacent genes.

Adjacent gene	Full name of the gene	Full name of the gene	Number of the target CpG cites	DNA positions of the target CpG cites in bears (NCBI sequence ID, position)	DNA region including the target CpG sites in bears++ (NCBI sequence ID, position)	DNA region corresponding to one in bears§ (humans: CpG ID; dogs or cats: chromosome (Chr), NCBI sequence ID, position)
GSE1	*GSE1*	Genetic Suppressor Element 1	4	NW_-020656126.1, 35404606, 35404611, 35404630, and 35404634	NW_-020656126.1, 35404570-35404839	cg07082267 (human) [4] Chr 5, NC_006587.3, 67230026-67230305 (dog) [1]
VGF	*VGF*	VGF Nerve Growth Factor Inducible	3	NW_-020656161.1, 15693978, 15693980, and 15693982	NW_-020656161.1, 15693842-15694006	cg21186299 (human) [4] Chr 6, NC_006588.3, 8701204-8701368 (dog) [1]
SLC12A5	*SLC12A5*	Solute Carrier Family 12 Member 5	4	NW_-020656136.1, 29314689, 29314692, 29314694, and 29314698	NW_-020656136.1, 29314538-29314961	cg07547549 (human) [4] Chr 24, NC_006606.3, 33293646-33294073 (dog) [1]
SCGN	*SCGN*	Secretagogin, EF-Hand Calcium Binding Protein	2	NW_-020656151.1, 1536251+ and 1536248+	NW_-020656151.1, 1536332-1536064+	cg06493994 (human) [4], [5] Chr 35, NC_006617.3, 23602291-23602560 (dog) [1]
KCNK12	*KCNK12*	Potassium Two Pore Domain Channel Subfamily K Member 12	3	NW_-020656123.1, 69049881+, 69049878+, and 69049876+	NW_-020656123.1, 69049936-69049754+	cg27320127 (human) [5] Chr 10, NC_006592.3, 49672242–49672424 (dog) [1]

Adjacent gene	Full name of the gene	Full name of the gene	Number of the target CpG cites	DNA positions of the target CpG cites in bears (NCBI sequence ID, position)	DNA region including the target CpG sites in bears++ (NCBI sequence ID, position)	DNA region corresponding to one in bears§ (humans: CpG ID; dogs or cats: chromosome (Chr), NCBI sequence ID, position)
OTUD7A	*OTUD7A*	OTU Deubiquitinase 7A	2	NW_-020656149.1, 5545866 and 5545874	NW_-020656149.1, 5545802-5546094	cg01763090 (human) [4] Chr 3, NC_006585.3, 37548040–37548332 (dog) [1]
BCL6B	*BCL6B*	BCL6B Transcription Repressor	6	NW_-020656175.1, 1898079+, 1898076+, 1898074+, 1898071+, 1898068+, and 1898065+	NW_-020656175.1, 1897691-1898117	cg10137837 (human) [4] Chr 5, NC_006587.3, 32051030–32051450 (dog) [1]
POU4F2	*POU4F2*	POU Class 4 Homeobox 2	4	NW_-020656132.1, 35281618+, 35281612+, 35281610+, and 35281608*	NW_-020656132.1, 35281323-35281738	cg05991454 (human) [4] Chr 15, NC_006597.3, 45028967–45029372 (dog) [1]
ELOVL2	*ELOVL2*	Elongation Of Very Long Chain Fatty Acids Protein 2	3	NW_-020656151.1, 13437488+, 13437486+, and 13437483+	NW_-020656151.1, 13437542-13437426+	cg16867657 (human) [6] Chr B2, NC_018727.3, 17965149-17965265 (cat) [2]
RALYL	*RALYL*	RALY RNA Binding Protein Like	5	NW_-020656127.1, 44864373+, 44864356+, 44864354+, 44864351+, and 44864347*	NW_-020656127.1, 44864394-44864286+	cg00034076 (human) [7] Chr F2, NC_018740.3, 34400393-34400501 (cat) [2]

Adjacent gene	Full name of the gene	Full name of the gene	Number of the target CpG cites	DNA positions of the target CpG cites in bears (NCBI sequence ID, position)	DNA region including the target CpG sites in bears++ (NCBI sequence ID, position)	DNA region corresponding to one in bears§ (humans: CpG ID; dogs or cats: chromosome (Chr), NCBI sequence ID, position)
KISS1R	*KISS1R*	KISS1 Receptor	1	NW_-020656189.1, 418487	NW_-020656189.1, 418311-418608	cg23989053 (human) Chr 20, NC_006602.3, 57758954* (dog) [3]
CAPS2	*CAPS2*	Calcyphosine 2	2	NW_-020656133.1, 29957817+ and 29957807+	NW_-020656133.1, 29957836-29957690+	cg12145547 (human) Chr10, NC_006592.3, 15889094 (dog) [3]

+ denotes the reverse complement.

++ DNA region including the target CpG sites in bears that was found to be homologous to DNA sequences of dogs or cats using BLAST.

§ Humans: CpG sites corresponding to or adjacent to target CpG sites in bears. Dogs or cats: DNA position or DNA region containing a target corresponding to the CpG sites in bears.

Reference: [1] Ito et al. 2017, [2] Qi et al. 2021, [3] Yamazaki et al. 2021, [4] Florath et al. 2014, [5] Day et al. 2013, [6] Bekaert et al. 2015, [7] Lowe et al. 2018

Table 2. Mean absolute error (MAE), median absolute error (MedAE), and root mean square error (RMSE) values for each model.

Model	Used CpGs	LOOCV model	LOOCV model	LOOCV model	wild bea
		MAE	MedAE	RMSE	MAE
Single regression	SL-4	1.582	1.174	2.095	1.735
Elastic net regression	SL-2, -3, -4, P-2, V-2, -3, SC-1	1.522	1.195	2.082	1.460
	SL-4, P-3, V-2, SC-2	1.591	1.294	2.087	1.297
	SL-4, P-2, V-2, SC-1	1.543	**1.048**	2.022	1.322
	SL-4, P-2, V-2	**1.485**	1.309	**1.979**	**1.242**
	SL-4, P-2	1.536	1.125	2.063	1.533
	SL-1,2,3,4	1.593	1.169	2.170	1.630
Support vector regression	all (13 CpG)	1.350	1.081	1.766	1.377
	SL-4, P-3, V-2, SC-2	1.504	1.294	1.863	1.397
	SL-4, P-2, V-2, SC-1	1.459	1.000	1.912	1.062
	SL-4, P-2, V-2	1.493	1.174	1.843	**1.020**
	SL-4, P-2	1.327	1.255	**1.608**	1.706

Model	Used CpGs	LOOCV model	LOOCV model	LOOCV model	wild bea
	SL-1, -2, -3, -4	**1.304**	**1.000**	1.622	1.610

All values were rounded to the fourth decimal place.

In the CpGs, SL, P, V, and SC represent SLC12A5, POU4F2, VGF, and SCGN, respectively.

+Values were generated by assigning methylation levels to each model. "Wild bears" did not include samples that were used for model establishment.

Table 3. Coefficients and p values for the linear regression of Δage in the SVR model.

	Estimate	P-value
(Intercept)	-0.02944	
Age	0.01474	0.4560
Growth environment (wild)	-0.01432	0.7227
Age: Growth environment (wild)	-0.1588	**0.00108**

Table 4. Coefficients and p values for the linear regression of |Δage| in the SVR model.

	Estimate	P-value
(Intercept)	0.08804	
Age	0.01422	0.2306
Growth environment (wild)	0.07705	**0.00238**
Age: Growth environment (wild)	0.05311	0.05642

Figure Legends

Figure 1. Map of Hokkaido, Japan, showing Noboribetsu Bear Park and the Rusha area of the Shiretoko Peninsula.

Figure 2. Scatter plots of age (year) versus DNA methylation level (%) in CpG sites that showed the strongest correlation. This figure includes one of the CpGs adjacent to*SLC12A5* (a), *POU4F2* (b), *VGF* (c), and *SCGN*(d).

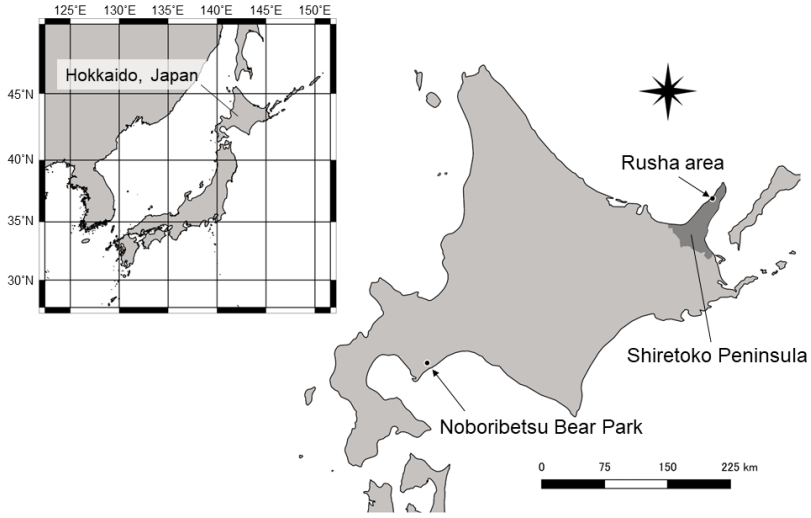
Figure 3. Within-individual changes in DNA methylation levels with age. The same CpG sites as in Figure 2 are shown. Dotted line represents the straight line approximated from the samples used for model construction. This figure includes one of the CpGs adjacent to *SLC12A5* (a), *POU4F2*(b), *VGF* (c), and *SCGN* (d).

Figure 4. Left: scatter plots of predicted age (year) and chronological age in the age estimation model after LOOCV. Right: scatter plots of predicted age (year) and chronological age for wild bears based on the methylation levels assigned to the left model. Solid line represents predicted age = actual age; distance between the dotted line and solid line represents the MAE of the model after LOOCV; single regression model (a, d), the best elastic net regression model (b, e), and the best SVR model (c, f).

Figure 5. Influences of the interactions among age, sex and growth environment on the model. (a) Scatter plots of Δage (year) and chronological age (year) in the female and male datasets. (b) Scatter plots of Δage (year) and chronological age (year) in the captive and wild datasets. The interaction between age and growth environment was significantly explanatory for Δage. (c) Scatter plots of |Δage| (year) and chronological age

(year) in the female and male datasets. (d) Scatter plots of |Δage| (year) and chronological age (year) in the captive and wild datasets.

Figure 6. Influences of sex and growth environment on the model: (a) Δage according to sex: female and male. (b) Δage according to growth environment. (c) |Δage| according to sex. (d) |Δage| according to growth environment. The growth environment was significantly explanatory for |Δage|.



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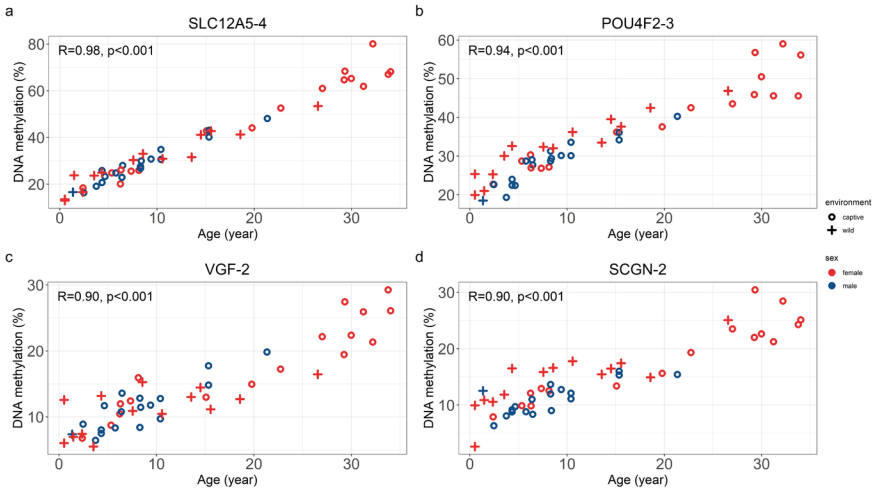

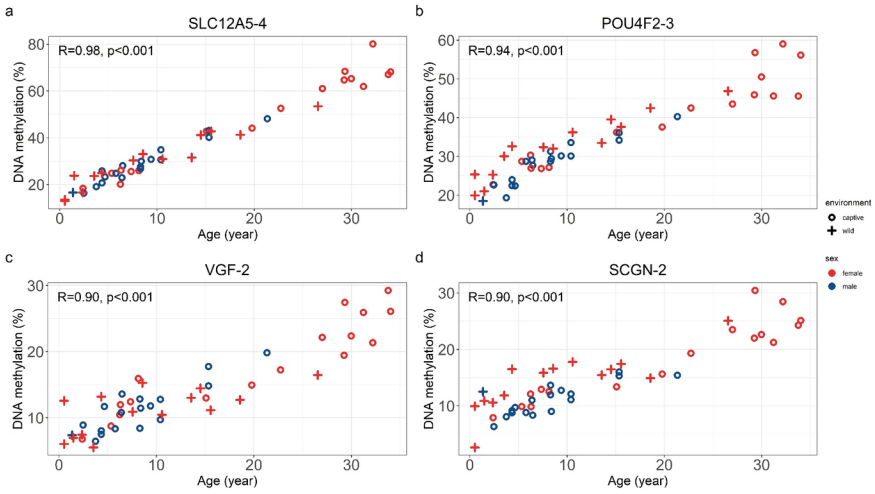

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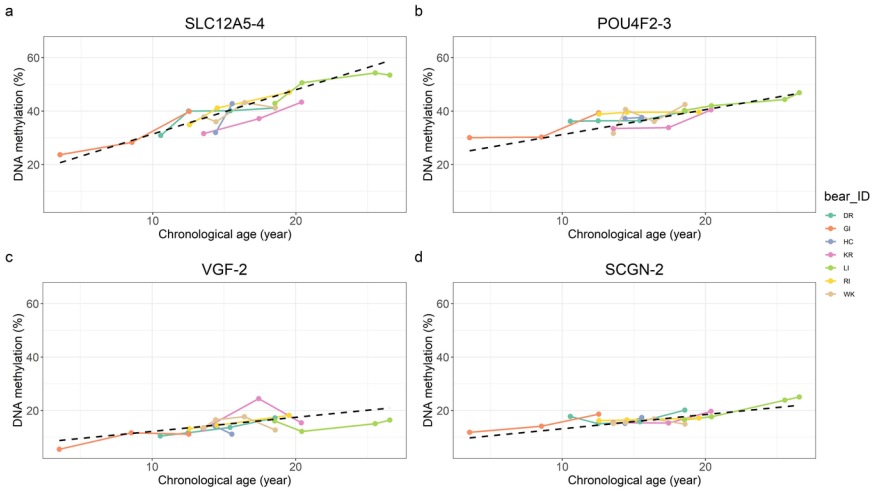

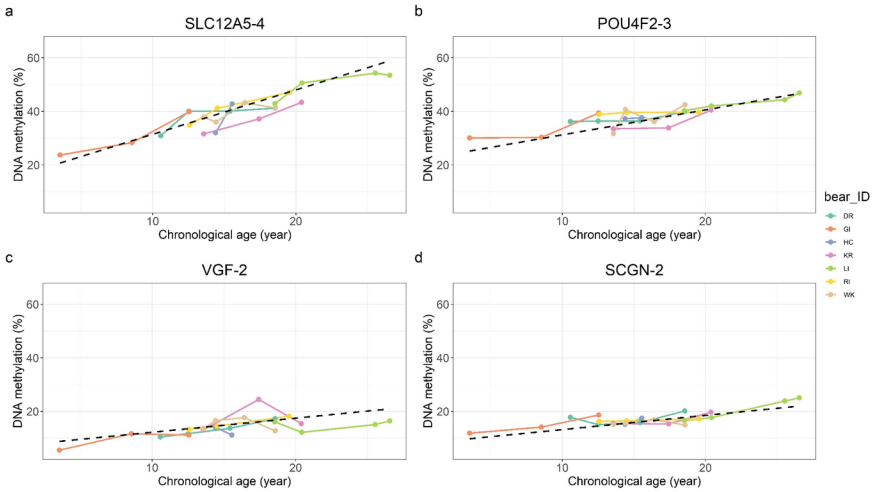

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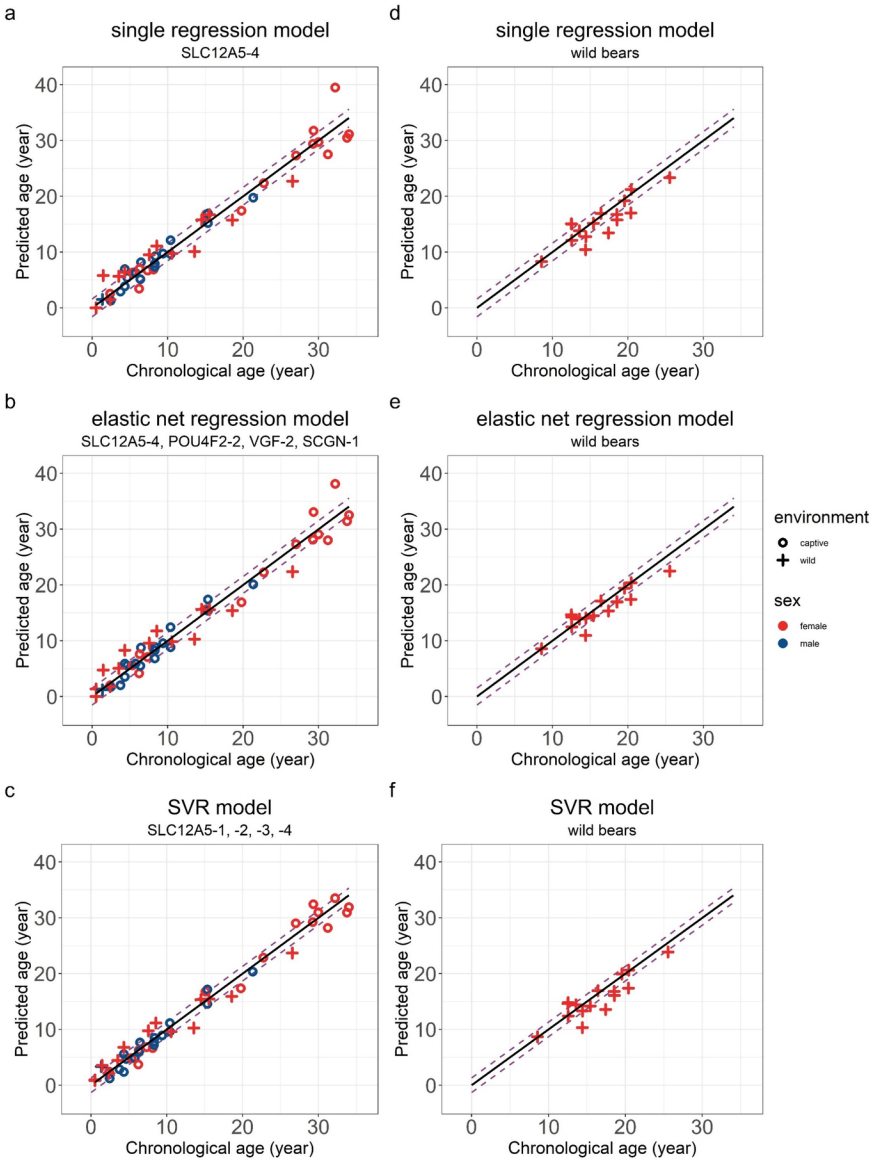

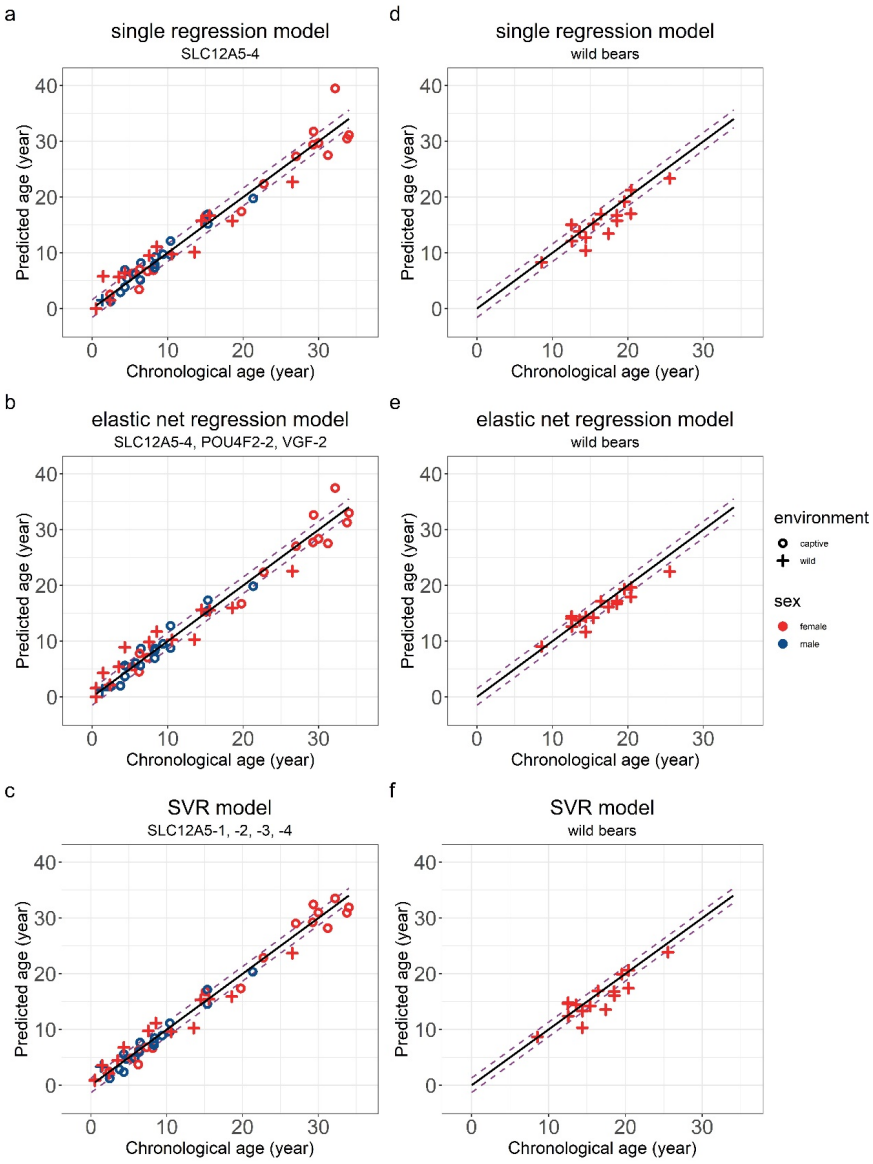

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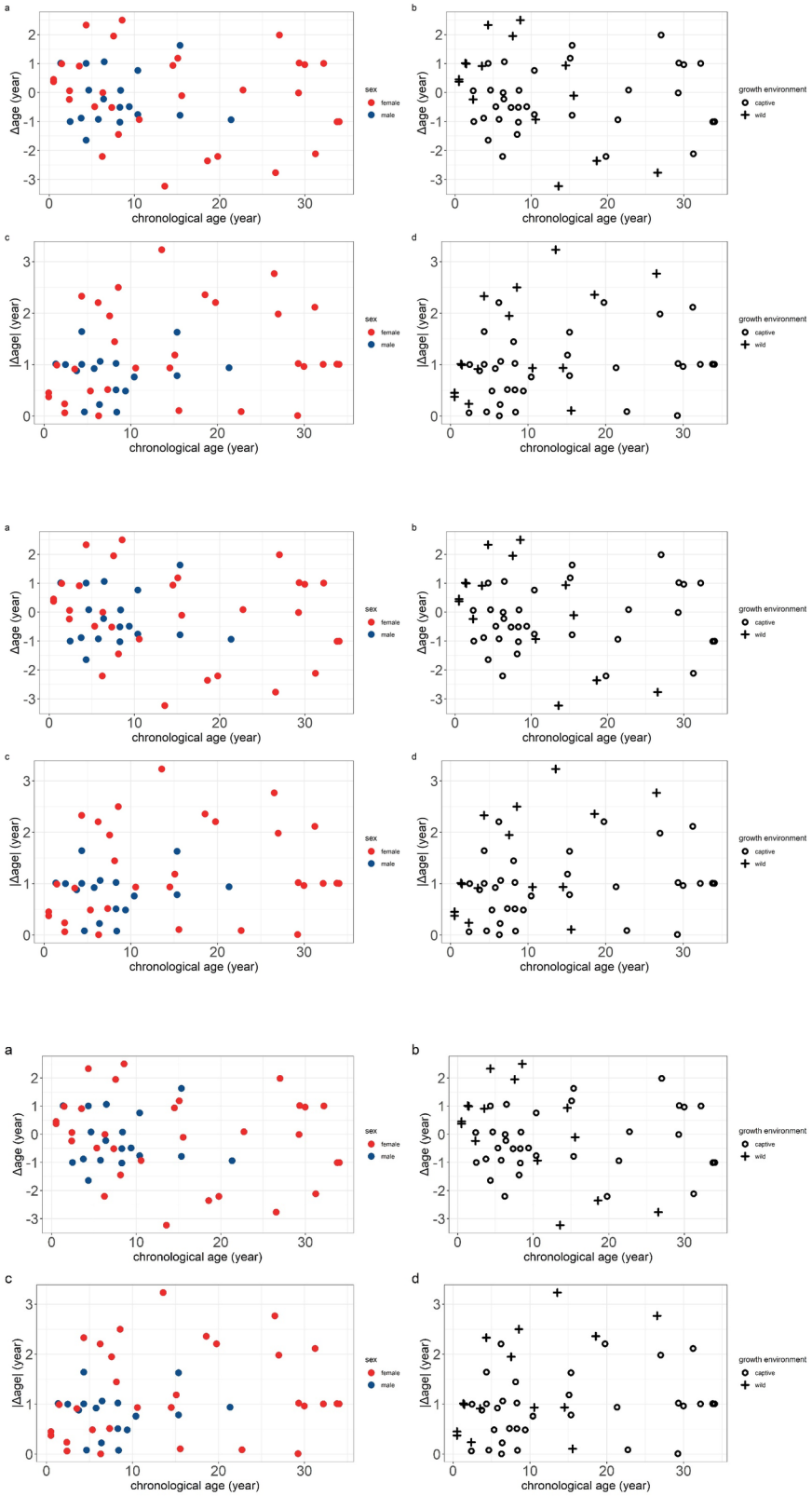

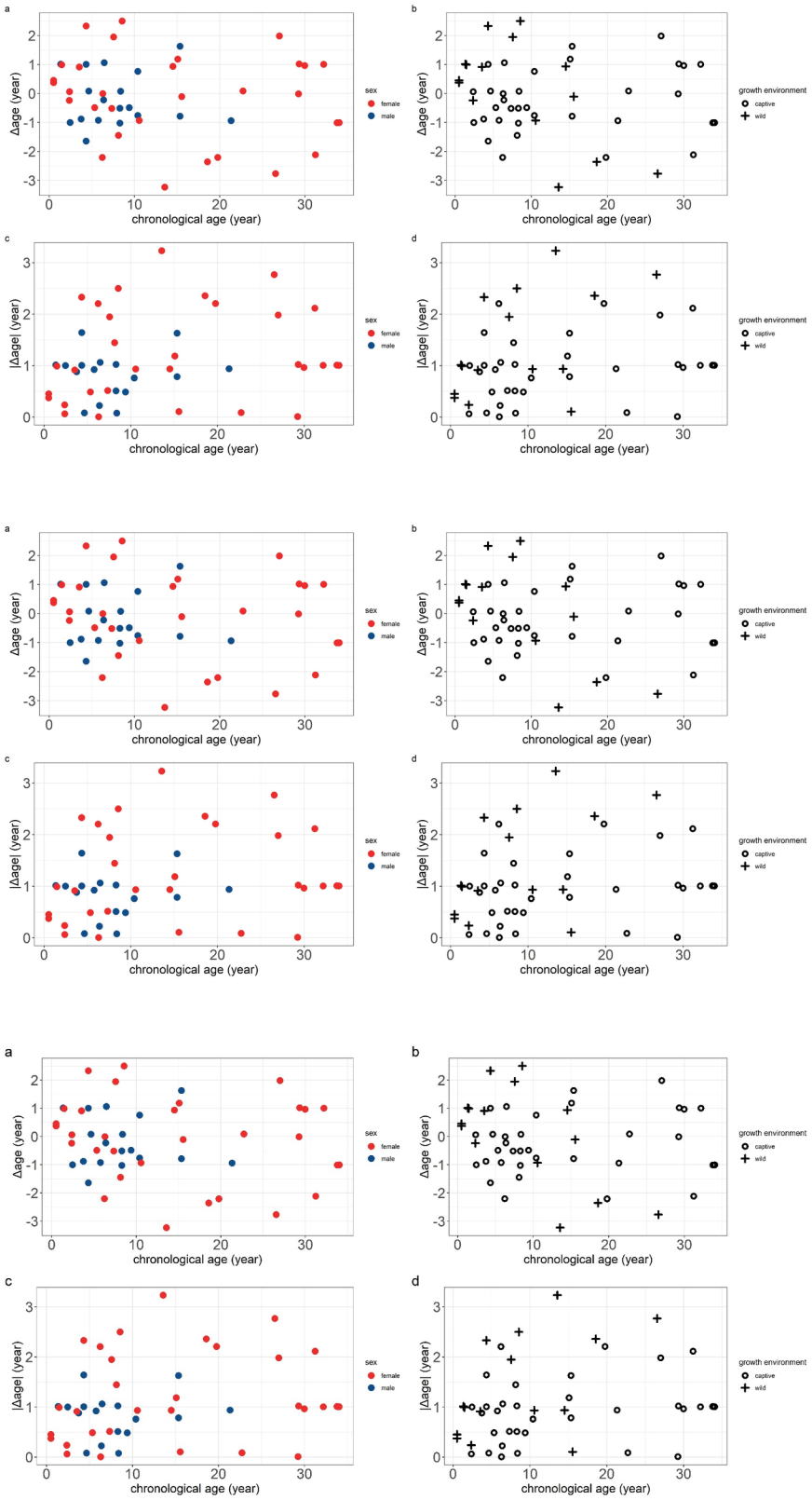

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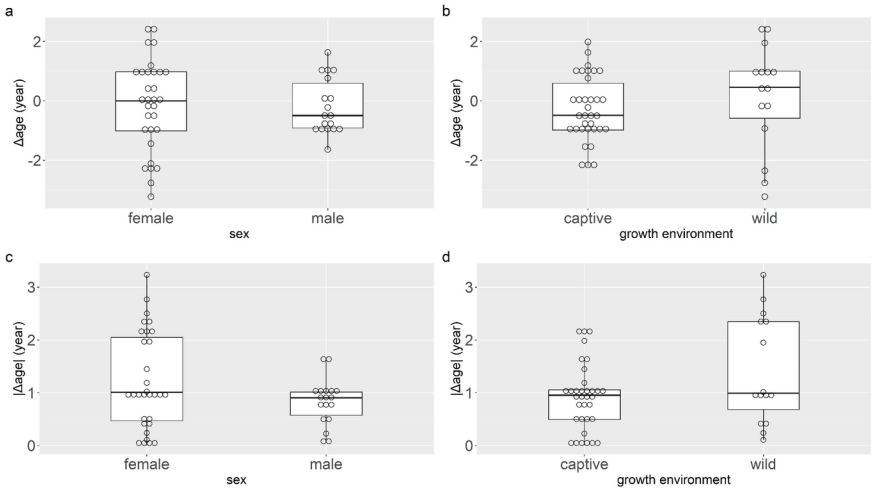

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