Isotope analysis of birds' eye lens provides early-life information

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Abstract

Early-life environment has a long-lasting effect on later life, though its estimation is often prevented in the wild because of a lack of available methods. Recently, isotope analysis of eye lenses has attracted considerable interest as a means to reconstruct the environmental conditions experienced by animals during the developmental period. This analysis has mostly been confined to fish for practical reasons and remains to be resolved for application to other animals. In this study, we broadened its applicability by developing a novel approach and verifying its

usability for the reconstruction of early-life environments. We performed a feeding experiment using Japanese quail (*Coturnix japonica*), in which we administered two diets: one composed mainly of C3 plants (low δ^{13}C and high δ^{15}N) and the other of C4 plants (high δ^{13}C and low δ^{15}N). Quails in the control group were continuously fed a C3-based diet from hatching until 200 days old, whereas those in the treatment groups (T10, T15, T20, and T40) were switched from the C3 to the C4-based diet at 10, 15, 20, and 40 days after hatching, respectively. We found that the δ^{13}C in the eye lenses of the treatment groups decreased from the center layer to the middle layer of the lens and then increased toward the outer layer, thus reflecting the diet change. In contrast, those of the control group exhibited a decreasing trend and equilibrated at the middle layer of the eye lens, with no increase thereafter. This novel approach revealed the postnatal feeding histories of the diet-shift experiment. The high δ^{13}C values observed in the center of the eye lenses would reflect the prenatal feeding environment, i.e., the C4-based diet consumed by their mothers, which is further reinforced by higher δ^{15}N values at this position due to the consumption of egg yolk-derived nutrition. These results indicate that the avian eye lens can be used as an "isotopic chronicle," which is a useful tool for reconstructing chronological isotopic information about their early-life history.

Keywords

Eye lens, Feeding experiment, Chronology, Stable isotope, Japanese quail

1 Introduction

Early-life environment has a long-lasting effect on the later life of animals and is known as the "silver spoon hypothesis" (Monaghan 2008). Food availability, habitat quality, or physiological stress during early life can affect future development, survival, and reproduction (Carlisle et al. 2015; Hayward et al. 2013; Hamel et al. 2009; Nussey et al. 2007). Understanding the early-life environments of organisms is important in pure science to clarify the exact causality of phenotypic development as well as in applied science for the conservation of declining species (Sergio et al. 2022). However, early-life information of grown animals is often challenging to obtain because continuous long-term research is required to track the same individual from natal to adult period, and thus a retrospective approach for the reconstruction of early-life environments is required.

Isotope analysis is a widely used technique for investigating the trophic status and migratory origins of organisms across numerous taxa (Hobson and Wassenaar 2018). For example, isotope analysis plays a pivotal role in elucidating the ecological dynamics of long-distance migrants, such as birds, because of the challenges associated with tracking their

migratory patterns and dietary preferences (Inger and Bearhop 2008; Tzadik et al. 2017). Inert integumentary tissues, such as bird feathers, have often been used as exemplary tissues for retrospective isotope analysis (Hobson and Clark 1992; Wolf et al. 2013), as they provide information on their development. In particular, isotope analysis of feathers has played an important role in avian ecology by explaining migratory patterns with a relatively small cost when compared to other methodologies, such as geo-loggers and GPS tracking (Hobson et al. 2012; Guiterrez-Exposito et al. 2015).

However, feathers and other inert integumentary tissues are not flawless because information may be lost after their renewal, i.e., after molting (Carvalho et al. 2022). Therefore, these analyses cannot provide permanent information about early-life environments, such as their birthplaces. Early-life information, which is particularly important, needs to be collected by other, more permanent inert tissues.

One tissue that can potentially preserve the environmental conditions experienced during early life is the eye lens, as eye lenses are composed of metabolically inert proteins that are sequentially deposited (Vecchio and Peebles 2020). Previous studies considering fish eyes indicated that eye lenses can be a valuable tool for reconstructing past environments. However, unlike fish and mammals, avian eye lenses are challenging to analyze because of their softness and water solubility (Simpson et al. 1994); therefore, there is a need to develop a method for

avian eye lens analysis.

Avian lens maturation follows a pattern that is common to vertebrates, with new fibers continuously emerging from the equatorial epithelial cells (Sivak 2004). The avian eye lens develops during embryonic stages, and it is postulated that only the outermost lens harbors post-embryonic information (Peebles and Hollander 2020). In addition, during the embryonic stage, synthesized proteins are transported to the aged and metabolically inactive cells at the center of the lens, referred to as the organelle-free zone (OFZ) (Shestopalov and Bassnett 2000), which can be challenging to clarify via isotope analysis of the early-life environment. On the other hand, unlike fish, the avian eye lens does not grow larger for more than 50 days, and also does not undergo compaction at the center of the lens (Augusteyn 2008). Thus, once we can develop a protocol to analyze avian eye lenses, a relatively stable lens size would make a useful tool to reconstruct the developmental environment. By establishing a diet-shift experiment in early life, we can assess whether the eye lens reveals the diet history and, if any, develop a formula to reconstruct the history of the early-life environment from the isotopic information while considering the substantial mixing of the isotopes in the radial direction (i.e., diffusion by the above-mentioned biological transport process and sample preparation process) (Passey and Cerling 2002).

The purpose of this study is to determine whether the environment experienced during

the early-life stage can be reconstructed from an avian eye lens. To achieve this, we performed a feeding experiment on captive Japanese quails (*Coturnix japonica*) for 200 days, using diets derived from C3 and C4 plants. After hatching, chicks received a C3-based diet (hereafter C3 food). The C3 food of the control group was maintained throughout the experiment, while the treatment groups switched to a C4-based diet (hereafter C4 food) at specific points (Fig. 1). Following the feeding period, we analyzed a time series of the isotopic ratios in both the eye lens and primary feathers. Based on the data, we aim to (1) test whether birds' lenses record the chronological isotopic information and (2) determine the duration of such information retention. Furthermore, we investigate the correspondence between the position in the eye lens and the age at which the information is deposited by developing a formula.

2 Methods/Experimental

2.1 Diet-shift experiments

For the C3 food (Kusanagi Farm Limited Company, Obihiro, Japan), food residues from potato peels, confectionery waste, cotton and pumpkin seeds, sake lees, and other sources were utilized. This mixture was fermented with lactic acid bacteria and combined with wheat, soybeans, yams, scallops, rice bran, starch flour, fishmeal, and beet cake (Mori et al. 2020). In contrast, the C4 food employed a commercially available mixed feed (Runkeeper, Marubeni Nisshin Feed Co.,

145 Ltd., Japan) (Goto et al. 2023), containing imported corn and additional components (Mori et
146 al. 2020). Feeding trials for both diets were performed at a dedicated facility in the Obihiro
147 University of Agriculture and Veterinary Medicine between June 1 and October 20, 2019.

148 Japanese quail eggs were collected from parent birds that were raised on the above-
149 mentioned C4 food (Runkeeper) (Goto et al. 2023) and were incubated at 36.5°C until hatching
150 (n = 31). Hatched chicks were housed individually and fed C3 food for 10 days. They were then
151 divided into control (n = 6) and treatment (n = 25) groups. The control group was fed C3 food
152 throughout the trial. The treatment group switched to the C4 food at specific time points: 10
153 days (T10; n = 6); 15 days (T15; n = 10); 20 days (T20; n = 7); and 40 days (T40; n = 2) after
154 the start of rearing. The final rearing period for the treatment groups included time points at 40,
155 80, 120, 160, 168, and 200 days (Table 1). The control group's final rearing periods were 17,
156 40, 80, 120, 160, 168, and 200 days.

157

158 **2.2 Eye lens dehydration and dissection**

159 To perform the continuous dissection of the fragile avian eye lens, which is susceptible to
160 liquefaction at room temperature, we established the following dehydration and drying protocol,
161 that was modified from the method described by Matsubayashi et al. (in preparation):

162 1. After a minor incision in the sclera with scissors, the frozen eyeballs were immersed in 10

163 mL of absolute ethanol within a screw-capped plastic tube on ice. This initial cut enabled
164 ethanol access to the eye lens while maintaining its structural integrity.

165 2. Following immersion for 10 min, the eyeball was extracted, the scleral incision was
166 enlarged, and the eyeball was returned to fresh absolute ethanol. This process was repeated
167 with incisions made along the equatorial plane of the sclera. This step enhanced surface
168 dehydration compared to the initial immersion because of the improved ethanol penetration.

169 3. Thirty minutes after the initial immersion, the eyeball was removed from the ethanol, and
170 the eye lens was carefully extracted using a spatula, tweezers, and scissors. It was then
171 placed in fresh absolute ethanol on ice for 30 min, followed by 60 min at room temperature.
172 This ensured the through dehydration of the entire eye lens through continued ethanol
173 exposure.

174 4. For complete internal eye lens desiccation, the eye lenses were dried overnight at 40°C in a
175 dedicated drying oven (DO-600FPA, As ONE, Osaka, Japan). The 40°C drying temperature
176 was selected to adjust to the general body temperature of quails (Underwood et al. 1999).
177 The dried eye lens was then dissected according to the protocol described by Matsubayashi
178 et al. (in preparation):

179 1. The lens was positioned on a Petri dish, photographed under a dissecting microscope (Leica
180 M125, Leica Microsystems GmbH, Wetzlar, Germany) equipped with a Moticam S3 camera

(Shimadzu Rika Corporation, Tokyo, Japan), and its diameter was measured from the circumference.

2. The lens was moistened with a minimal amount of deionized water. Using fine-tipped tweezers, the soft material was carefully collected from across the entire surface and transferred to a new designated container.
3. After scraping off approximately 0.5–200.0 μg of eye lens sample for isotope analysis while preserving the overall lens shape, any remaining water was wiped away, and the used instruments were washed or replaced as required.
4. The above steps 1–3 were repeated until the central core of the eye lens was reached.

Because of the inherent aspherical nature and unevenness of the ethanol-dehydrated quail ocular lenses, the short axis was used as the basis for size determination. This defines the lens size as the shortest diameter at the equator (mm). Ethanol treatment for 2 h should not have a significant effect on the stable isotope analysis of epithelial tissue or amino acids, including the eye lens (Borrow et al. 2008; Chua et al. 2020). The analysis excluded the outermost lens section containing the attached fibrae zonulares (Fitzgerald 2018). This discrepancy between lens size and the outermost analyzed position may be due to this exclusion. Finally, individual lens sections were freeze-dried using a freeze dryer (FDU-2100, EYELA, Tokyo, Japan). We excluded samples of the 160-day-old control quail because of sample degradation.

199

200 **2.3 Feather processing**

201 As the first primary feathers had already completely grown by 40-days old, we only analyzed
202 the first primary (P1) feathers (Paritte and Kelly 2009; Wolf et al. 2013) from 40, 120, and 160-
203 day-old birds (control, T10, T15, and T20). The samples were washed with water, soaked in a
204 2:1 chloroform: methanol solution for 24h, and then rinsed twice with methanol to remove any
205 surface contaminants and oils. After being air-dried in a fume food for more than 24h (Paritte
206 and Kelly 2009), the samples were moved to a drying oven (DO-600FPA, As ONE, Osaka,
207 Japan) at 60°C for more than 24h. The feather vanes were cut using surgical scissors from the
208 tip to the base, approximately 2–3 mm each but approximately 10 mm for the narrow feather
209 tip, which was sufficient for isotope analysis.

210

211 **2.4 Stable isotope analysis**

212 We performed continuous dissection of the fragile avian eye lenses that are susceptible to
213 liquefaction and placed them along with 0.05–0.16 mg of feathers and lens sections, and 0.65
214 mg of C3 food, and 0.86 mg of C4 food into tin capsules for the carbon and nitrogen stable
215 isotope analyses. Isotope ratios were measured using a Delta V Advantage mass spectrometer
216 (Thermo Fisher Scientific, Waltham, MA, USA) connected to a Flash EA 2000 elemental

analyzer via a Conflo IV interface (Thermo Fisher Scientific). The stable carbon ($^{13}C/^{12}C$) and nitrogen ($^{15}N/^{14}N$) isotopic compositions of the sample (δ_{sample}) were expressed in standard delta (δ) notation according to the following formula (Couplen 2011):

$$\delta_{sample} = (R_{sample}/R_{std}) - 1$$

where R_{sample} is the corresponding isotope ratio $^{13}C/^{12}C$ or $^{15}N/^{14}N$ for the sample. R_{std} is the defined isotope ratio by international standard scales, where $^{13}C/^{12}C$ is the Vienna Pee Dee Belemnite (VPDB) for $^{13}C/^{12}C$ and atmospheric nitrogen for $^{15}N/^{14}N$. The isotope ratios of carbon and nitrogen were calibrated against those of the DL-alanine ($\delta^{13}C$ = –25.36‰, $\delta^{15}N$ = –2.89‰; CERKU-01), L-alanine ($\delta^{13}C$ = –19.04‰, $\delta^{15}N$ = 22.71‰; CERKU-02), and L-threonine ($\delta^{13}C$ = –9.45‰, $\delta^{15}N$ = –2.88‰; CERKU-05) laboratory standards, which were calibrated against international standards (Tayasu et al. 2011). The analytical standard deviations (SDs) of the DL-alanine standard (n = 53) were 0.09‰ ($\delta^{13}C$) and 0.04‰ ($\delta^{15}N$); those of the L-alanine standard (n = 50) were 0.10‰ ($\delta^{13}C$, n = 51) and 0.06‰ ($\delta^{15}N$, n = 56); and those of the threonine standard (n = 54) were 0.12‰ ($\delta^{13}C$) and 0.07‰ ($\delta^{15}N$).

2.5 Data analysis

To investigate the correspondence between the position in the eye lens and the age at which the information is deposited, we developed the following model, which has as its starting point a

235 piecewise constant function (PCF), which represents the signature in the eye lens of the

236 switching of diets from the maternal diet (C4 food) to the initial diet (C3 food) and then back

237 to C4 food. This function was convoluted with a normalized Gaussian profile with a standard

238 deviation σ to model the mixing of isotope ratios in the radial direction, which could be caused

239 by contamination of layers with different time frames during the sample preparation or through

240 biological transport process. The convolution evaluates to:

241 $$\delta = \delta_1 + \frac{1}{2}(\delta_1 - \delta_2)\left[\mathrm{erf}\left(\frac{\frac{r_\mathrm{h}}{1+ca} - r}{\sqrt{2}\sigma}\right) - \mathrm{erf}\left(\frac{\frac{r_\mathrm{h}+r_\mathrm{T}}{1+ca} - r}{\sqrt{2}\sigma}\right)\right]$$

242 where $\mathrm{erf}\,(\cdot)$ denotes the conventional error function; δ_1 is the asymptotic value for C4 food,

243 i.e., the value where an isotopic equilibrium state is achieved after a change in diet (Ayliffe et

244 al. 2004); δ_2 is the asymptotic value for C3 food; a is the age of quails at the time of

245 dissection; r_h is the radius corresponding to the time of hatching; c is the rate of lens

246 compaction; r_T is the radius corresponding to the start of the C4 feeding period ($r_T = \infty$ for

247 the control); and r is the actual distance from the lens center.

248 We fitted this model to the complete δ^{13}C dataset (Supplementary data by minimizing

249 the mean squared difference between δ^{13}C observed at radius r and the model prediction of δ

250 by adjusting the model parameters σ, δ_1, δ_2, a, r_h, c, and the four independent parameters

251 of r_T corresponding to the four diet switching times at T =10, 15, 20 and 40 days. From this,

252 we estimated the transition lens radius ($r_\mathrm{h} + r_\mathrm{T}$) for each of the dissection date (Supplementary

Figure S3). We fitted two model variants, with and without ($c = 0$) lens compaction. In the eye lens of vertebrates, there is a mechanism called compaction, in which the central part of the lens shrinks and does not become larger (Augusteyn 2008), which we account for through the c parameter. To date, no studies have examined whether compaction occurs over time in the avian eye lens. Therefore, we constructed models both with and without the compaction parameter (c) and compared them using Akaike information criterion (AIC).

To confirm whether quail lens sizes increased with the dissection date, the relationship between lens size and age (17– 200 day old) was analyzed using a linear model (LM). The isotopic offset between the tissue and the diet (e.g., $\delta^{13}C_{lens-diet}$) was calculated by subtracting the isotope ratios of the diet from those of the equilibrated tissue.

For the $\delta^{15}N$ isotope ratio, the asymptotic value was also estimated by following the PCF model:

$$\delta = \frac{1}{2}\left[(\delta_1 + \delta_3) + (\delta_1 - \delta_2)\,\mathrm{erf}\left(\frac{\frac{r_h}{1+ca} - r}{\sqrt{2}\sigma}\right) + (\delta_2 - \delta_3)\,\mathrm{erf}\left(\frac{\frac{r_\mathrm{h} + r_\mathrm{T}}{1+ca} - r}{\sqrt{2}\sigma}\right)\right]$$

where δ_1 is the asymptotic value of maternal nutrition, i.e., a high value should be assumed by bioenrichment irrespective of food contents; δ_2 is the asymptotic value for C3 food; δ_3 is the asymptotic value for C4 food. The other numerical statements are the same as the above-mentioned PCF model for $\delta^{13}C$. All data analyses were performed using the R statistical computing environment version 4.1.2 (R Core Team, 2021).

3 Results

3.1 Stable isotope ratios of diet and eye lens

The $\delta^{13}C$ values of the C3 and C4 food were −26.4‰ and −17.1‰, respectively. The PCF model, including compaction, provided a better fit (AIC = 804.5) over the model without controlling for lens compaction (AIC = 837.3, Supplementary Fig. S1). Therefore, the pattern of isotope shift was inferred from the model with compaction (Supplementary Table S1, showing all fitted parameters). In particular, the control quails initially had a high $\delta^{13}C$ (average ± SD = −19.7‰ ± 1.4‰, with a range of −18.4 to −22.1‰), which decreased with the lens radius (i.e., age), and stabilized at −23.9‰ ± 0.2‰ (Supplementary Table S1). The treatment groups exhibited a peak $\delta^{13}C$ value in the innermost lens sections, which then dropped and rose again towards the outer sections (Fig. 2). Treatment groups achieved maximum $\delta^{13}C$ values in the outermost lens sections, which approached the equilibrated $\delta^{13}C$ value of the C4 food (Supplementary Fig. S1, −17.6‰ ± 0.3‰). Individual variation in $\delta^{13}C$ existed even among quails with the same rearing conditions (Fig. 2).

The $\delta^{15}N$ values of the C3 and C4 foods were 4.6‰ and 2.1‰, respectively. The PCF model that included the compaction parameter c provided a better fit (AIC = 388.5) compared to the model without c (AIC = 416.9, Supplementary Fig. S2). Therefore, the isotope shift

pattern was inferred from the model with compaction (Supplementary Table S2, showing all fitted parameters). The $\delta^{15}N$ values in the center of the eye lens were approximately 5.7‰ ± 0.1‰, which decreased with age and reached equilibrium at 4.5‰ ± 0.1‰ (Supplementary Table S2, Fig. 3(a)). The offset of $\delta^{15}N$ between the C3 food (4.6 ‰) and eye lens ($\delta^{15}N_{lens\text{-}diet}$) was estimated to be −0.1 ‰. The treatment group also showed a $\delta^{15}N$ shifts (i.e., decrease) from the center to outer eye lens sections, achieving equilibrium at 3.9‰ ± 0.1‰ (Supplementary Fig. S2, Supplementary Table S2), with individual variations, especially at the innermost sections (Fig. 3). The offset of the $\delta^{15}N$ between the C4 food (2.1 ‰) and eye lens ($\delta^{15}N_{lens\text{-}diet,}$ 3.9 ‰) was estimated to be 1.8 ‰.

3.2 Lens size and compaction

The mean lens size was 1.00 ± 0.07 mm at 17 days (n = 2), 1.23 ± 0.13 mm at 40 days (n = 8), 1.21 ± 0.15 mm at 80 days (n = 8), 1.22 ± 0.15 mm at 120 days (n = 6), 1.34 ± 0.11 mm at 160 days (n = 7), 1.24 ± 0.07 mm at 168 days (n = 3), and 1.10 ± 0.13 mm at 200 days (n = 7). There was no significant increase in the eye lens radius with age (from 17 days to 200 days old, n = 41, LM: Coef. ± SE = 0.000082 ± 0.0004, t = 0.21, p = 0.83). From the PCF models, the lens compaction rate was estimated to be 0.0012 mm per day for the $\delta^{13}C$ dataset (Supplementary Fig. S3(a), Supplementary Table S1) and 0.0013 mm per day for the $\delta^{15}N$ dataset

307 (Supplementary Fig. S3(b), Supplementary Table S2).

308

309 **3.3 Stable isotope ratios of primary feathers**

310 We determined the $\delta^{13}C$ and $\delta^{15}N$ values of the P1 feathers from quails at 40 days, 120 days,

311 and 160 days (control, T10, T15, and T20 for each). The feather $\delta^{13}C$ values in the control group

312 remained almost constant throughout the feather section (Fig. 4, mean ± SD = −23.6‰ ± 0.2 ‰,

313 n = 3). In the treatment group, all 40-day-old quails exhibited lower $\delta^{13}C$ values in the tip

314 sections, which increased with growth and reached equilibrium around the $\delta^{13}C$ value of the C4

315 food (Fig. 4). The other birds showed almost constant $\delta^{13}C$ values (Fig. 4, −16.7‰ ± 0.4 ‰).

316 The feather $\delta^{15}N$ values of the 120 and 160-day-old quails in the control and treatment

317 groups exhibited individual differences but remained consistent within the individuals (mean ±

318 SD; control group: 3.9‰ ± 0.5‰, treatment groups: 3.4‰ ± 0.5‰, Supplementary Fig. S3). In

319 the two 40-day-old quails, the initial $\delta^{15}N$ value was high and decreased as they grew, eventually

320 reaching equilibrium around the similar value of the 120 and 160-day-old quails of the same

321 group.

322

323 **3.4 Trophic discrimination factors (TDF) of the quail eye lens and primary feather**

324 We estimated the trophic discrimination factors (TDFs) (i.e., the offset) between each food and

tissue. The offset of $\delta^{13}C$ values between the eye lens (−23.9‰ for the constant value of the control group) and the C3 food ($\delta^{13}C_{lens\text{-}diet}$) was estimated to be 2.5‰ (Supplementary Table S1). The offset of $\delta^{13}C$ values between the eye lens (−17.6‰ for the constant value of the treatment group) and the C4 food ($\delta^{13}C_{lens\text{-}diet}$) was estimated to be −0.5‰ (Supplementary Table S1). The offset of $\delta^{15}N$ values between the eye lens and the C3 food ($\delta^{15}N_{lens\text{-}diet}$) was estimated to be −0.1 ‰ (4.5‰, Supplementary Table S1). The offset of $\delta^{15}N$ values between the eye lens and the C4 food ($\delta^{15}N_{lens\text{-}diet}$) was estimated to be 1.8‰ (3.9‰ for constant value of treatment group, Supplementary Table S1).

The offset of the $\delta^{13}C$ values between the feather (−23.6‰ for the constant value of the control group, Fig. 4 (a)) and the C3 food ($\delta^{13}C_{feather\text{-}diet}$) was estimated to be 2.8‰ (Supplementary Table S1). The offset of the $\delta^{13}C$ values between the feather (16.7‰ for the constant value of the treatment group, Fig. 4 (b)−(d)) and the C4 food ($\delta^{13}C_{feather\text{-}diet}$) was estimated to be 0.4‰. The offset of the $\delta^{15}N$ values between the feather and the C3 food ($\delta^{15}N_{feather\text{-}diet}$) was estimated to be −0.7 ‰ (3.9‰ for the control group, Supplementary Fig. S4 (a)). The offset of $\delta^{15}N$ values between the feather and the C4 food ($\delta^{13}C_{feather\text{-}diet}$) was estimated to be 1.3‰ (3.4‰ for the constant value of the treatment group, Supplementary Fig. S4 (b)−(d), Supplementary Table S1).

4 Discussion

In this study, we found that the early-life environment could be reconstructed using the eye lens from quails. The high $\delta^{13}C$ values observed in the center of the eye lens for all individuals may reflect the isotope ratios of yolk-derived nutrition, specifically the C4 food fed to their mother (Fig. 2). Subsequently, in the control group of quail that was continuously fed the C3 food, the $\delta^{13}C$ values in the eye lens transitioned to lower values and equilibrated. In the quails of the treatment groups, the transition to lower C3 food-derived $\delta^{13}C$ was followed by a shift to higher C4 food-derived values, and this U-shaped shift was preserved in the quails at all switching times (10, 15, 20, and 40 days, Fig. 2). Thus, we confirmed the diet shifts up to 40 days, and hence the quail eye lens should retain isotopic information for at least 40 days after hatching. Furthermore, the decreasing $\delta^{13}C$ shift from the center to the outer lens was consistent within the control group from 40- to 200-day-old quails (Fig. 2), indicating that the early-life environment is conserved for at least 200 days.

To estimate the timing at which the most recent isotopic information is obtained from the avian eye lens, it is necessary to understand their growth pattern. No significant increase in lens radius was found in individuals after 40 days of age, which is supported by the previous studies in which the avian eye lens did not enlarge after 50 days of age (e.g., house sparrows, *Passer domesticus*, Augusteyn, 2014). Even if lens growth does not occur after its size reaches

361 the asymptote, lens compaction allows for the further preservation of isotopic records
362 (Augusteyn 2008). In our study, the PCF model of both $\delta^{13}C$ and $\delta^{15}N$ datasets indicated the
363 presence of lens compaction (Supplementary Fig. S3), but a previous study reported that the
364 absence of lens compaction in chickens (*Gallus domesticus*) has also been observed (Augusteyn
365 2014). In this study, the longest time to diet shift was 40 days; thus, to determine how long the
366 isotopic record can be stored, it is necessary to conduct experiments with a longer time to diet
367 shift.

368 We also revealed differences in the isotope ratios between feathers, which have been
369 the most commonly used tissue for isotopic studies in birds (Hobson and Wassenaar 2018), and
370 the eye lens, which we propose as a new target for retrospective isotope analysis. Unlike eye
371 lenses, feathers could not recover embryonic $\delta^{13}C$ records (Fig. 4), thereby indicating that lenses
372 can recreate the early-life environment, which cannot be obtained from feathers. In addition, in
373 feathers, the transitions from C3 to C4 foods were reflected only in the isotope ratios of quails
374 in the 40-day-old treatment group but not in the 120- and 160-day-old groups, indicating that
375 their early-life environment was lost due to molting. This can be a major obstacle in inferring
376 the isotope ratios (Hobson and Wassenaar 2018). On the other hand, feathers provide isotopic
377 information during feather development, which can be directly linked to information on feather
378 growth. Thus, we showed that the sequential isotope analysis of avian eye lenses provides a

379 new method to complement information on their prenatal phase that is challenging to obtain
380 from feathers.

381 Although the feeding trial of the current study was designed primarily for $\delta^{13}C$, the
382 results of $\delta^{15}N$ generally supported those of $\delta^{13}C$ (Fig. 3). The higher $\delta^{15}N$ values near the center
383 of the eye lens can be explained by the trophic enrichment associated with the consumption of
384 egg yolk-derived nutrients (Hobson1995). When purposely analyzing $\delta^{15}N$ of the eye lens to
385 understand the trophic ecology via the relationship between animals and their food, this effect
386 of trophic enrichment should be considered. The continuous decline reflecting the diet shift
387 from C3 food to C4 food (Fig. 3) indicated that the avian eye lens also preserves a chronological
388 record of $\delta^{15}N$ from the prenatal to early postnatal periods. For the $\delta^{15}N$ of feathers, there was
389 a large variation in the isotope ratios at equilibrium, stored period, and the direction of isotopic
390 ratio among individuals (Supplementary Fig. S4). These irregularities may be linked to feather
391 wear, such as mechanical abrasion and UV light causing a loss of melanin (Johnson 2019), and
392 the source of amino acids, such as endogenous reserves and fed proteins (Cherel et al. 2005),
393 but they do not affect the conclusions obtained for $\delta^{13}C$.

394 The TDFs between the food and tissue in the target organisms are important
395 information when using isotope analysis in ecological studies, which can help predict the
396 isotopic composition of the diets (Hobson and Clark 1992). Because this factor is tissue-

397 dependent, it is worthwhile to estimate the TDFs of eye lenses and feathers to reconstruct the
398 history of diet consumption in a comparable form (Martinez del Rio et al., 2009). The TDFs in
399 quail eye lens and feathers determined in this study (C3 food: $\delta^{13}C_{lens\text{-}diet}$ = 2.5‰, $\delta^{13}C_{feather\text{-}diet}$
400 = 2.8 ‰, Supplementary Table S1) were similar to those of feathers from a total of 25 bird
401 species ($\delta^{13}C_{feather\text{-}diet}$ = 2.2‰, Swan et al. 2019), but higher than those of whole blood (0.4‰,
402 Swan et al. 2019). The TDFs based on $\delta^{15}N$ in the current study (C3 food: $\delta^{15}N_{lens\text{-}diet}$ = −0.1‰,
403 $\delta^{13}C_{feather\text{-}diet}$ = −0.7‰, Supplementary Table S1) were estimated to have a lower value than
404 those of previous studies ($\delta^{15}N_{feather\text{-}diet}$ = 4.1‰, Swan et al. 2019, Supplementary Table S1).
405 This lower TDF estimation may partly be related to the difference in the reflection speed of the
406 isotope ratio of the components that are used as a carbon and nitrogen resources via exogenous
407 (mixed feed) and endogenous (stored nutrients) sources depending on their developmental stage
408 (Ayliffe et al. 2004). Furthermore, the mixed feed used in the current study included multiple
409 food sources; therefore, variation in the chemical characteristics (carbon chain length, number
410 of double bonds, degree of unsaturation) between the food components could affect digestibility
411 and energy use in quails (Valentim et al. 2023). These are unavoidable and an intrinsic problem
412 in the TDF estimation using mixed feed. Future research should consider the developmental
413 stages of birds and the turnover rate of their dietary sources (West et al. 2004; Ayliffe et al.
414 2004).

5 Conclusions

In the current study, we developed a methodology for the isotope analysis of avian eye lenses and validated its retrospective use for the reconstruction of early-life environments. Through diet-shift experiment, we were able to reconstruct the early-life history deposited in the avian eye lens and develop a new formula. Our method of dehydration by ethanol and dissection using fine-tipped tweezers is essential to prevent the disintegration of the avian lens. This method can be applied not only to pure science studies but also to applied studies for the reconstruction of the early-life environment for declining species and the implementation of conservation efforts. Although there were large individual differences in the isotope ratios within each treatment group, the control group converged to a single asymptotic value, indicating its reproducibility. Generally, hatchlings (both in precocial and altricial birds) remain in their birthplace, are not expected to disperse within a few weeks, and thus have sufficient time to store their early-life environment within their eye lens, thus making a birds' eye lens as a valuable tool to reconstruct their early-life environment. Further research is required to improve the accuracy and reproducibility of reconstructing the early-life environments left in the bird eye lens.

Abbreviations

Air: Atmospheric nitrogen; OFZ: Organelle-Free Zone; PCF: Piecewise Constant Function; TDFs: Trophic Discrimination Factors; VPDB: Vienna Pee Dee Belemnite

Declarations

Availability of data and material

The datasets supporting the conclusions of this article are available in the odf.io repository after acceptance.

Competing interests

The authors declare no competing interests.

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Authors' contributions

JM and EA contributed equally to this work and are co-first authors. JM and TA proposed the

topic and conceived and designed the study. TG, and HI performed the feeding trial. JM, EA, and IT developed the method for eye lens dissection and dehydration. EA, CY, JM, and IT performed isotope analysis. AGR developed the formula for data analysis. JM and EA wrote the initial draft of the manuscript. JM, EA, MH, AGR and IT collaborated on the data analysis and its interpretation and the construction of the manuscript. All authors have read and approved the final manuscript.

Authors' information

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Ethics statement

This study was approved by the Experimental Animal Committee of the Obihiro University of Agriculture and Veterinary Medicine (authorization number 19-32).

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575 ## Figure legends

576 Figure 1. Diet-shift experiment design. Japanese quail eggs were collected from parent birds
577 that were raised on C4 food. The hatched chicks were fed C3 food during the first 10 days, and
578 they were then divided into control group (n = 6) and treatment (total n = 25) groups. The
579 control group continued on the C3 food until the end of the experiment (i.e., dissection date at
580 days 17, 40, 80, 120, 160, and 200). The treatment group switched to the C4 food at specific
581 time points: 10 days (T10, n = 6), 15 days (T15, n = 10), 20 days (T20, n = 7), and 40 days
582 (T40, n = 2) after the start of rearing. The final rearing period (i.e., dissection date) for the
583 treatment groups included time points at 40, 80, 120, 160, 168, and 200 days.

584 Figure 2. ^{13}C values of quail eye lenses. (a) Control group (n = 5); (b) T10 group (n = 6); (c)
585 T15 group (n = 10); (d)T20 group (n = 7); and (e) T40 group (n = 2; 168 days). The lamina
586 from each eye lens (right or left) was included.

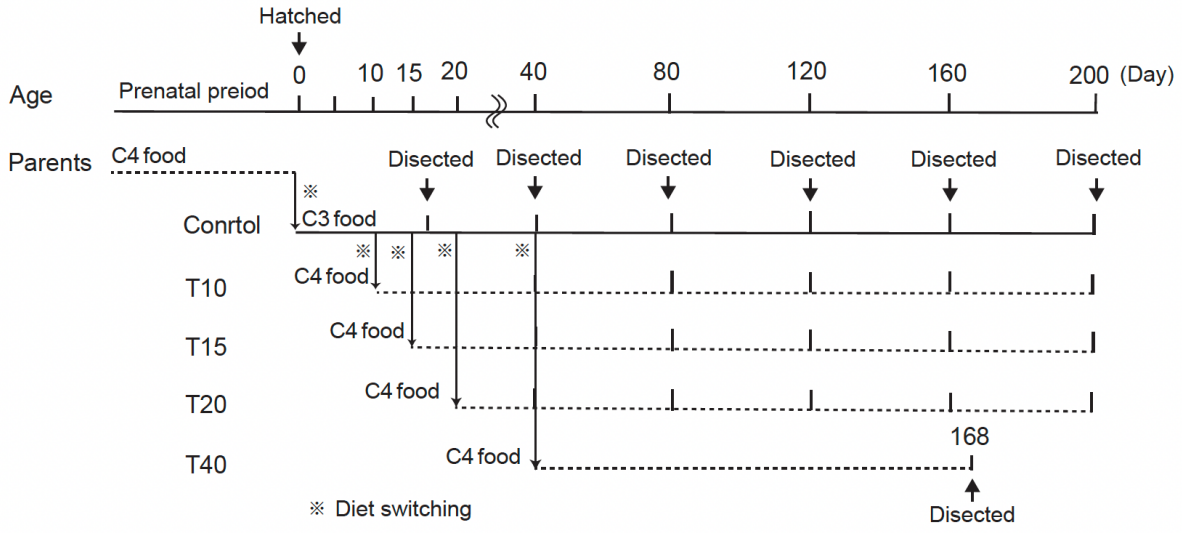
587 Figure 3. δ^{15}N values of quail eye lenses: (a) Control group (n = 5); (b) T10 group (n = 6); (c)
588 T15 group (n = 10); (d) T20 group (n = 7); and (e) T40 group (n = 2; 168 days). The lens
589 sections from each of eye lens (right or left) were included.

590 Figure 4. δ^{13}C values of quail feather sections. (a) Control group (n = 3); (b) T10 group (n = 3);
591 (c) T15 group (n = 3); and (d) T20 group (n = 3).

592

593 Fig.1

594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Age
Prenatal preiod
0
10
15
20
40
80
120
160
200
(Day)
Parents
C4 food
Disected
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Disected
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※
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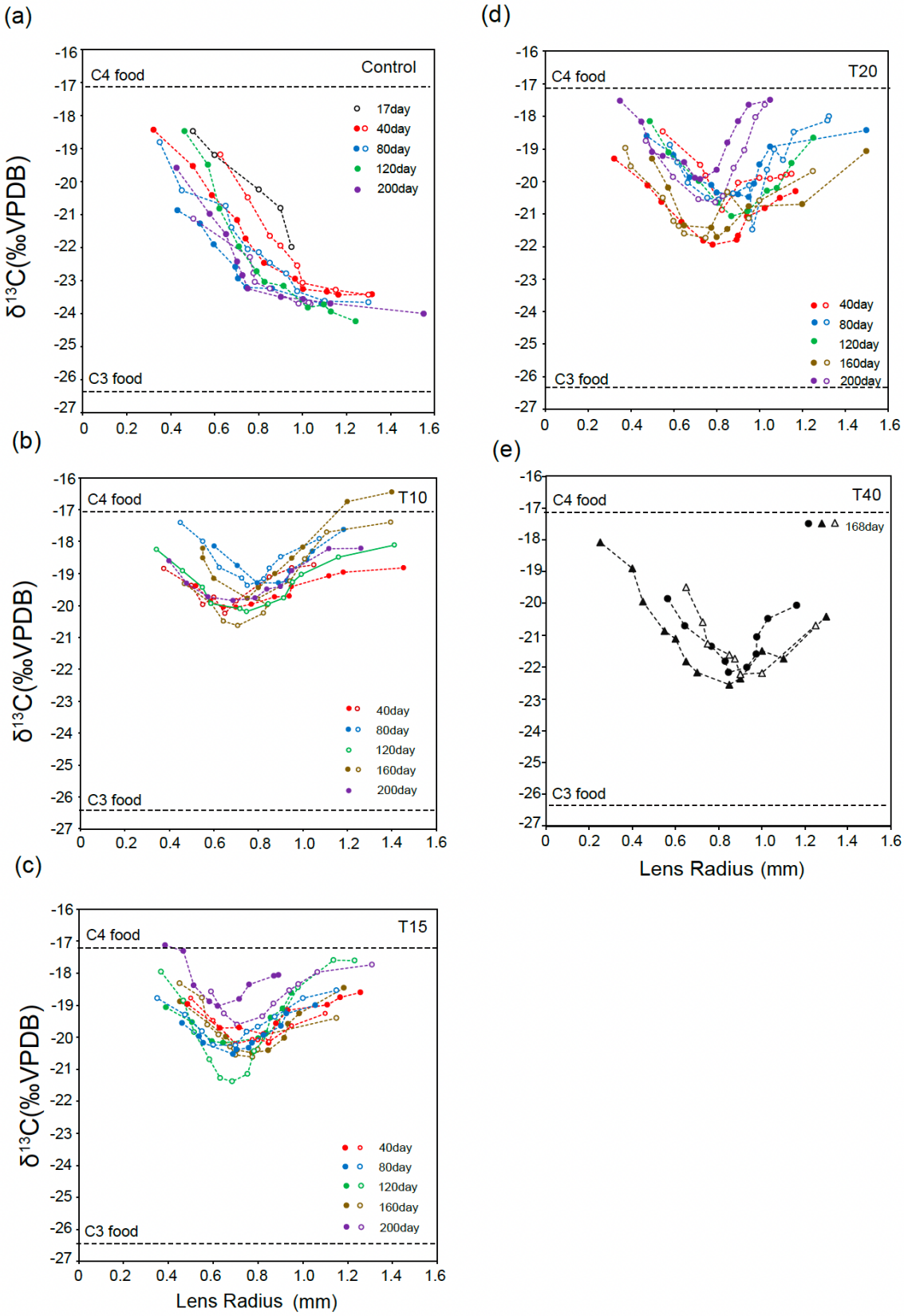
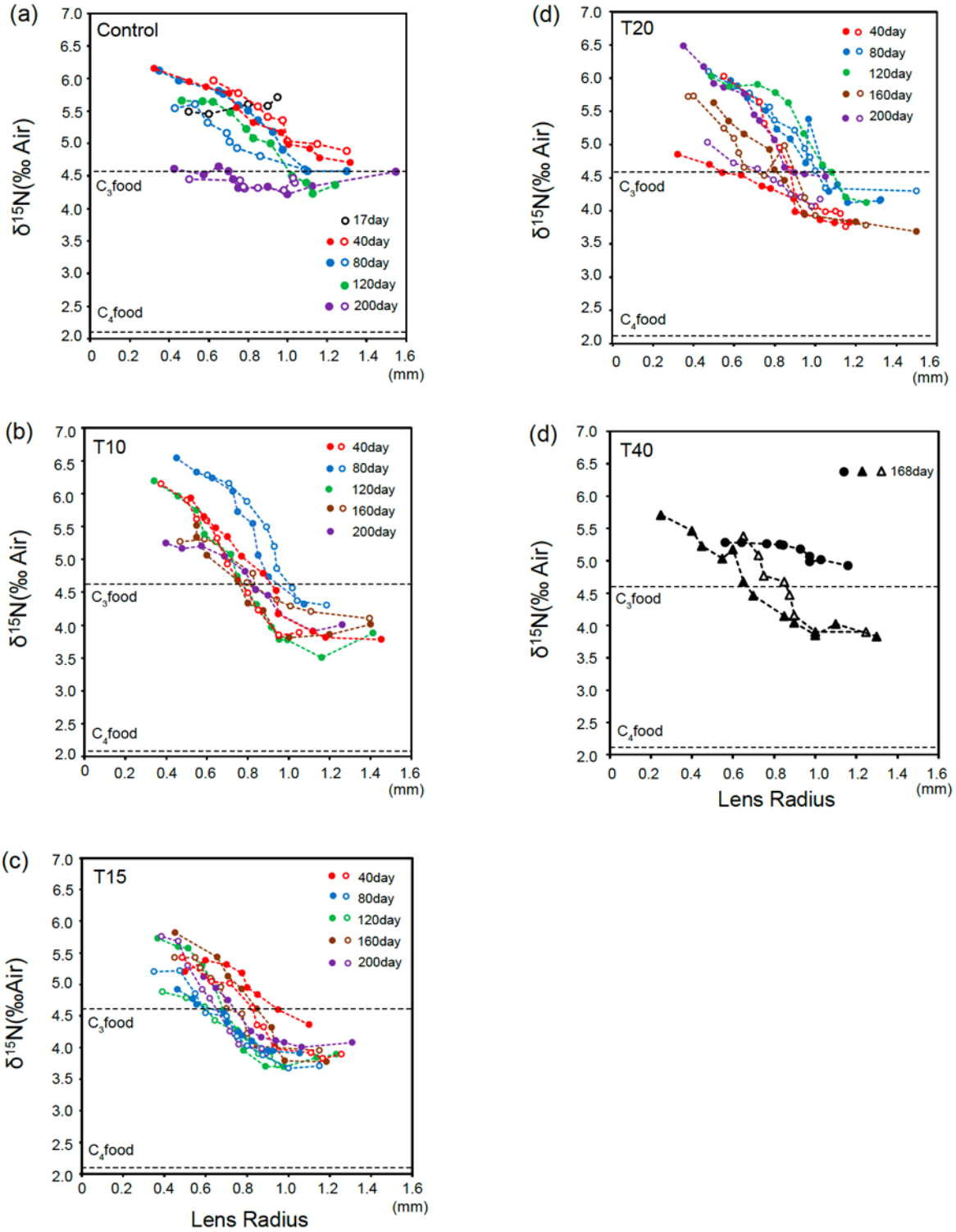
C4 food
T10
C4 food
T15
C4 food
T20
168
C4 food
T40
※ Diet switching
Disected

Fig.2


(a)
Control
C4 food
C3 food
17day
40day
80day
120day
200day
(b)
T10
40day
80day
120day
160day
200day
(c)
T15
(d)
T20
(e)
T40
168day
δ13C(‰VPDB)
Lens Radius (mm)

597 Fig.3

598


(a)
Control
17day
40day
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C3food
C4food
δ15N(‰ Air)
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T10
40day
80day
120day
160day
200day
C3food
C4food
δ15N(‰ Air)
(mm)
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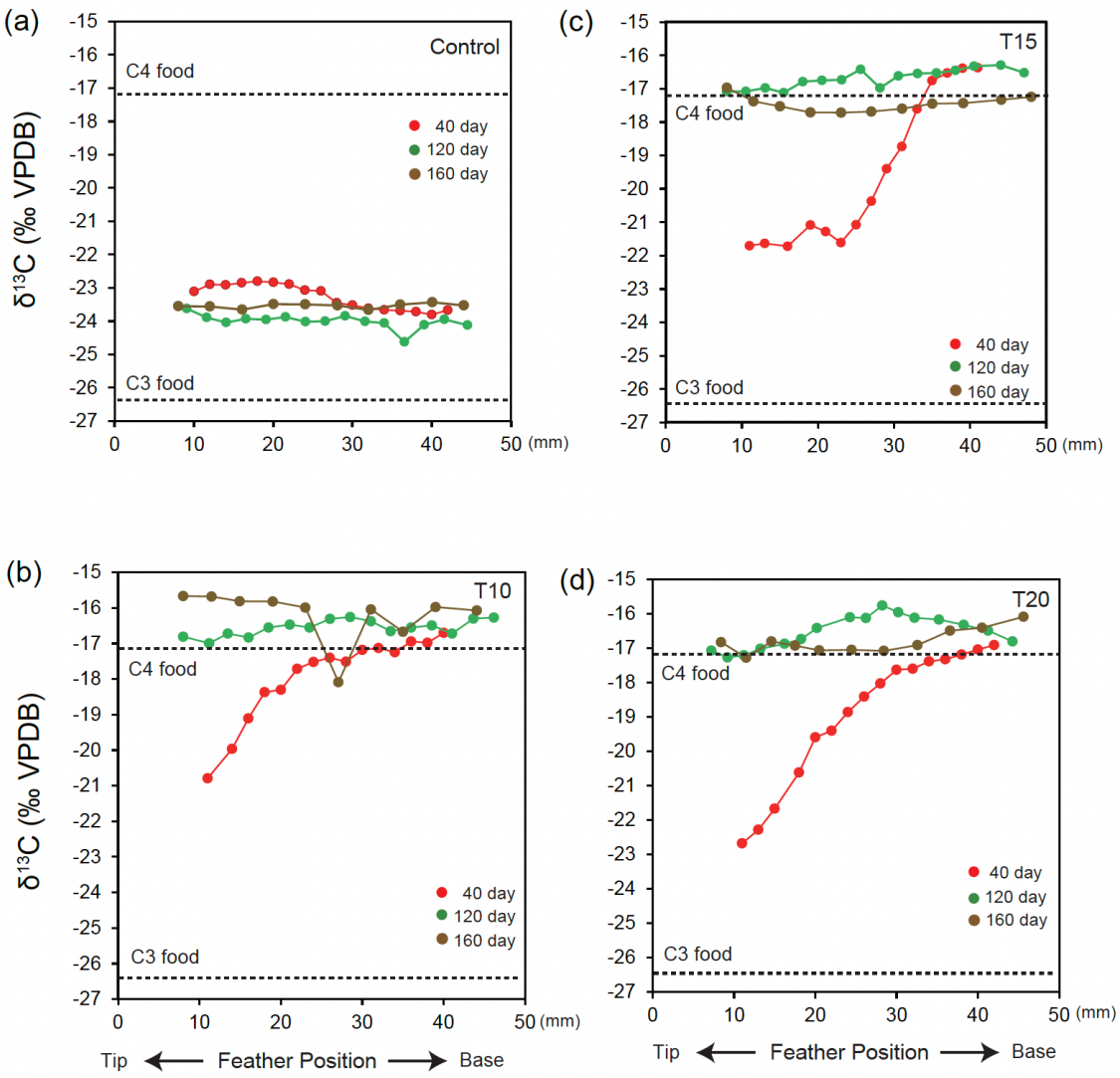
T15
40day
80day
120day
160day
200day
C3food
C4food
δ15N(‰Air)
Lens Radius
(mm)
(d)
T20
40day
80day
120day
160day
200day
C3food
C4food
δ15N(‰ Air)
(mm)
(d)
T40
168day
C3food
C4food
δ15N(‰ Air)
Lens Radius
(mm)

599 Fig.4

600


(a)
Control
C4 food
C3 food
40 day
120 day
160 day
δ13C (‰ VPDB)
50 (mm)
(c)
T15
C4 food
C3 food
40 day
120 day
160 day
50 (mm)
(b)
T10
C4 food
C3 food
40 day
120 day
160 day
δ13C (‰ VPDB)
50 (mm)
Tip
Feather Position
Base
(d)
T20
C4 food
C3 food
40 day
120 day
160 day
50 (mm)
Tip
Feather Position
Base