

Article

Hoof Growth Rates of the European Roe Deer (*Capreolus capreolus*) for Dating the Hoof's Isotopic Archive

Benjamin D. Hafner ¹ , Andreas König ² and Karl Auerswald ^{3,*} ¹ Technical University of Munich, Ecophysiology of Plants, Hans-Carl-von-Carlowitz-Platz 2, 85354 Freising, Germany; benjamin.hafner@tum.de² Technical University of Munich, Wildlife Biology and Management Unit, Hans-Carl-von Carlowitz-Platz 2, 85354 Freising, Germany; koenig@wzw.tum.de³ Technical University of Munich, Grassland Science, Alte Akademie 12, 85354 Freising, Germany

* Correspondence: auerswald@wzw.tum.de; Tel.: +49-8161-71-3242

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Abstract: Hooves preserve the isotopic information laid down during their growth and may be used for reconstruction of animal feeding history. To assign certain positions along hooves to corresponding times, growth rates are required. Hoof growth rates are known for domestic animals; however, they cannot be obtained easily in wild animals. We estimated the hoof growth rate of the European roe deer (*Capreolus capreolus* L.) by using the immediate drop in $\delta^{13}\text{C}$ along the hoof as a tag that is assigned to the date of maize (*Zea mays* L.) harvest. Keratin samples were taken each mm along 17 hooves and analyzed for their $\delta^{13}\text{C}$. A linear regression between (1) time differences of expected maize harvest to animal death and (2) distances between the points of the $\delta^{13}\text{C}$ drop to the periople yielded the growth rate. Mean hoof growth rate was 0.122 mm/day (95% CI 0.014 mm/day) and 0.365%/day ($\pm 0.026\%$ /day) of the hoof length and within the range of domestic animals. The method may be applied to determine growth rates of other incrementally growing tissues. Our estimated growth rate fosters dating isotopic information in hooves, facilitating research on feed resources and space use of roe deer.

Keywords: carbon; European roe deer; hooves; isotopic clock; nitrogen; stable isotopes; turnover time; ungulate

1. Introduction

Stable isotope analysis has become a major tool in the analysis of plant growth and vegetation change [1–3]. Tree rings allow retrospective views on growing conditions [4]. However, tissue of herbaceous species as found in the understory of forests is continuously replaced and does only provide a short temporal record. Furthermore, species replacement cannot be retrieved from tree rings. Animals feeding from herbaceous material record the isotopic information of their feed in their tissues [5,6]. Thus, they provide long-term records of short-lived vegetation. Furthermore, grazing or browsing integrates over the feeding grounds, removing small-scale heterogeneities between individual plants or plant organs [7]. Such heterogeneities can especially be expected for herbaceous species or young trees occupying niches. Stored animal tissues like wool products or hunting trophies provide long-term time series [8] but may also be used to resolve within growing periods [9].

As metabolic turnover rates of different animal tissues differ, isotope analysis of different tissues from the same animal allows reconstructing its feeding history, which is known as ‘isotopic clock’ [10]. Due to the continuous turnover of any living tissue, the isotopic clock is limited in its temporal precision. Incrementally growing, keratinous tissues such as horn [11], hair [12] or hooves [13–15]

offer the advantage that they do not turn over after formation and preserve the information laid down during their growth. As natural weathering does not change this information [16], it can be extracted even from archeological finds [17]. Incrementally growing tissues can be analyzed incrementally. Depending on their growth rate and their length, the isotopic feed composition can be retrieved with high temporal resolution (several days) over periods of several months (e.g., hooves or hair) to several years (e.g., hair [9] or horns [11]). However, to assign a certain position along an incrementally growing tissue to a certain time, usually two things have to be known: (1) the time when the tissue ceased growth (the time of sampling from a living animal or the death of an animal) and (2) the growth rate.

The European roe deer (*Capreolus capreolus* L.) is an even-toed ungulate whose weight is borne approximately equally by the third and fourth toes. It has a body length of 95–135 cm, a shoulder height of 65–75 cm, and a weight of 15–35 kg [18,19]. In the wild, roe deer attain a lifespan of 10 years [19]. Roe deer do not have horns but antlers that grow only during a short period of time and thus cannot provide a continuous nutrition record. Hairs are limited in suitability, too, because of the seasonal change between winter and summer fur, causing the hair growth rate to change seasonally. In contrast, hooves should grow more continuously because they are continuously worn down and they would allow retrieving isotope information over some months. The papillae (Figure 1A) are pushing the hoof wall downwards, therefore providing a chronological record of hoof growth from hoof entry to hoof tip. Due to the slanting arrangement of the papillae, deep-seated layers of the wall are slightly younger than superficial layers.

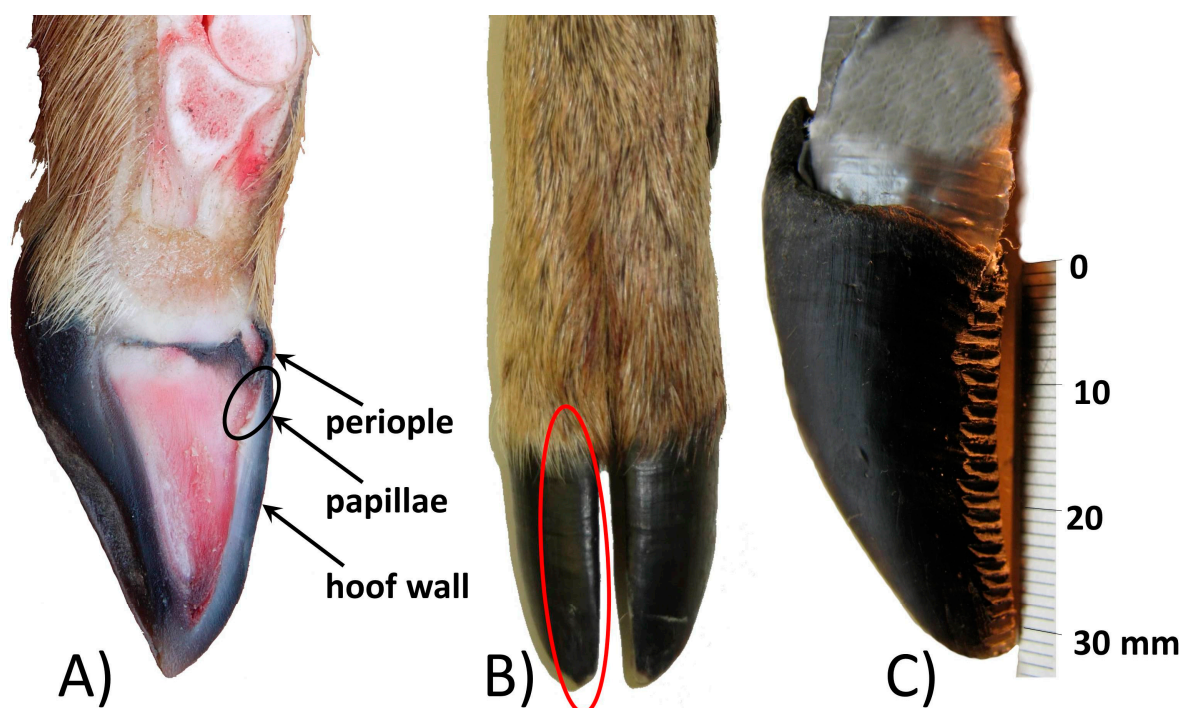


Figure 1. (A) Cross-section of a hoof; (B) Front view of a roe deer foot (oval denotes front edge of the hoof); (C) Side view of a hoof after sampling the front edge.

For roe deer, hoof growth rates are not known. While hoof growth rates can be obtained rather easily in domestic animals (e.g., by tagging a certain position at the hoof and recording the displacement of the tag over time [20]), this cannot be applied to wild animals. Wear and growth of hooves depend on running activity, soil surface properties and feed. Therefore, hoof growth rates determined on roe deer in captivity cannot simply be transcribed to wild animals.

To estimate the hoof growth rate of wild roe deer, we approached the possibility to assign a sudden change in $\delta^{13}\text{C}$ along the hoof to a known change in diet at a certain time. Maize (*Zea mays* L.)

is the only plant using the C4 photosynthetic pathway, which is of relevance north of the Alps. Farms growing maize have an intensive animal production or use maize for biogas. On such farms, usually high $\delta^{15}\text{N}$ values occur due to the large N surplus [21]. $\delta^{15}\text{N}$ in keratinous tissue may then reach values of up to 7‰, indicating feed from agricultural land. Maize disappears within a short period from the fields because the complete aboveground plant is preserved in silos for feeding purposes and silos have to be filled and closed quickly. Usually most of the maize is harvested within a fortnight in late October/early November [22]. After harvest of maize, roe deer must feed from plants using the C3 photosynthetic pathway. The large contrast in carbon isotopes between C4 and C3 plants (about 14‰ [23]) is recorded in the animal tissue [5,8]. $\delta^{13}\text{C}$ in keratinous tissue thus increases from about −26‰ with pure C3 diet to −12‰ with pure maize diet, which includes enrichment by 2.7‰ due to the diet-keratin shift [21]. The sudden change in the availability of maize should thus provide a tag in hooves that can be traced to estimate hoof growth rates in wild roe deer.

2. Materials and Methods

The study took place in the rural district Rottal-Inn in Eastern Bavaria [24], an area covered by a mosaic of about 30% spruce-dominated forests, 35% grassland, and 35% arable land, 20% of which was maize. Patch sizes of forests were about 25 ha while patch sizes of agricultural land were around 2.5 ha. We gathered roe deer samples following the official game harvest plan according to the German and Bavarian game harvest regulations. Revocations for hunting of animals during the closed season were issued by the regional game management authorities (Untere Jagdbehörden) on base of Art 33 (5) BayJG i.c.w § 22(1) 4 BJagdG [25]. In 2013 and 2014, 17 animals (four female and 13 male) were obtained in the period from mid-February to mid-May, during which we expected the maize peak to be detectable in the hooves (Table 1).

Table 1. Data obtained from 17 roe deer that were hunted between April 2013 and April 2014.

No.	Hoof Length (mm)	Samples	Sample Distance (mm)	Hunting Date	Day of the Year	Sex	Live Weight (kg)	Half-Life (day)
#207	38	38	1.0	20 February 2014	51	f	21.0	12.5
#231	34	19	1.8	1 March 2014	60	m	20.4	12.4
#212	33	23	1.4	2 March 2014	61	f	20.4	12.4
#216	39	26	1.5	8 March 2014	67	m	22.3	12.7
#225	30	21	1.4	14 March 2014	73	m	21.6	12.6
#222	34	18	1.9	3 April 2014	93	m	17.8	12.0
#118	32	23	1.4	10 April 2013	100	m	17.2	11.9
#119	37	19	1.9	10 April 2013	100	m	24.2	13.0
#228	37	19	1.9	16 April 2014	106	m	19.7	12.3
#128	35	19	1.8	17 April 2013	107	f	17.8	12.0
#131	32	17	1.9	1 May 2013	121	m	15.9	11.7
#132	35	25	1.4	1 May 2013	121	m	19.7	12.3
#134	37	20	1.9	2 May 2013	122	f	19.7	12.3
#136	34	18	1.9	5 May 2013	125	m	21.6	12.6
#138	31	29	1.1	14 May 2013	134	m	18.6	12.2
#141	32	24	1.3	17 May 2013	137	m	18.4	12.1
#142	48	22	2.2	19 May 2013	139	m	19.1	12.2
Minimum	30	17	1.0		51		15.9	11.7
Mean ± SD	35 ± 4	22 ± 5	1.6 ± 0.3		101 ± 29		19.7 ± 2.1	12.3 ± 0.3
Maximum	48	38	2.2		139		24.2	13.0

For each animal, the outer front claw of the right front foot was sampled immediately after hunting. The hoof wall was cleaned and removed from the claw by submersion into 65 °C warm water for some minutes and then dried. Lipid material covering the surface was removed with a methanol-ethanol mixture [12]. The dried hoof was sampled along the abaxial side of the anterior (front) edge (Figure 1B). This edge is almost straight and grows linearly, allowing an easy determination of sampling positions and thus position to time conversion. Further, the hoof wall (Figure 1A) is

thickest at this position and [15] have shown that growth rate of (bovine) hooves is slowest along the anterior side, which lets us expect that a long time record can be captured.

The front edges were 35 mm (SD 4 mm) long. Seventeen to 38 1-mm wide samples were taken per hoof, leaving small ridges between the sampled locations (Table 1). Sampling was done with a hacksaw, starting at the youngest part at the growing zone (perioplic ring or periople, Figure 1A). A 1 mm wide strip of approximately 3 mm length and 0.5 mm depth (Figure 1C) was filed off. The saw dust was immediately transferred into Eppendorf vials (Eppendorf AG, Hamburg, Germany) by a funnel located below the hoof. The funnel and the saw were cleaned after every sample by pressurized air. Then, pictures were taken to determine the position of every sample along the hoof, and for some hooves the left out ridges were sampled accordingly. Deviations of a position from the intended position were small (on average 0.12 mm) and not considered.

Rumen content was sampled directly after hunting, stored in a cooler and freeze-dried the day after. The freeze-dried material was homogenized and a subsample was ball milled for isotope analysis.

Between 250 µg and 350 µg of hoof saw dust or dried rumen content, respectively, were weighed into tin capsules, which were subsequently scrunched and transferred to a “0 blank” auto-sampler purged with He. Samples were analyzed for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ by combusting the samples in an elemental analyzer (NA 1110; Carlo Erba, Milan, Italy) interfaced (ConFlo III; Finnigan MAT, Bremen, Germany) to an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT). Carbon and nitrogen isotopic data are presented as $\delta^{13}\text{C}$ (‰) relative to the Vienna Pee Dee Belemnite standard and $\delta^{15}\text{N}$ (‰) relative to the air nitrogen standard:

$$\delta X = [(R_{\text{sample}}/R_{\text{standard}}) - 1] \times 1000 \text{ (‰)} \quad (1)$$

where R is the ratio of $^{13}\text{C}/^{12}\text{C}$ or $^{15}\text{N}/^{14}\text{N}$ and X is the corresponding element (C or N).

Measurement precision against the laboratory standard (horn meal, $\delta^{13}\text{C}$: -25.4‰ and $\delta^{15}\text{N}$: 5.7‰ , respectively) was better than 0.2‰ (SD) for $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$. To determine analytical precision including hoof heterogeneity and sampling error (position and depth of sampling), a second sampling was performed some weeks later on 21 positions of three hooves (Figure 2). Both samplings correlated with $R^2 = 0.967$ and the root mean squared error was 0.5‰ for $\delta^{13}\text{C}$, which mainly resulted from slight positional inaccuracies.

To estimate the hoof growth rate it was necessary to know the time between two sampling points of a known distance. The distances between the points were known from the regular distribution throughout the hoof sampling (Table 1). One point of time was also known from the hunting date (periople, first sample). The second date was determined via the signal decrease in $\delta^{13}\text{C}$ after the maize harvest (assumed on day of the year, DOY, 306; adopted from [22]). To identify the exact time point, the inflection point of the slope for the disappearance of the maize signal was chosen; that is, when half of the maize signal in the hoof had disappeared. To find the date of this point, we estimated the turnover rate of carbon in the animal based on its weight.

Carbon and nitrogen are delivered to the hoof from the blood plasma. Therefore, the stable isotope signal in the hoof depends on the turnover rate (half-life $t_{0.5}$) of the blood plasma. The plasma turnover for roe deer was extrapolated from data for blood plasma of cows (*Bos taurus* L. [26]) and rats (*Rattus norvegicus* Berkenhout [27]) as it is proportional to body mass [28]:

$$t_{0.5} = a_{\text{tissue}} \times M^{-0.25} \quad (2)$$

where a_{tissue} is a tissue-specific parameter and M is body mass. Knowing the half-lives and body masses for cow [26] and rat [27], we were able to calculate the turnover rate of blood plasma for roe deer by:

$$t_{0.5} \text{ (roe deer)} = \frac{M_{\text{roe deer}}^{-0.25}}{M_{\text{cow or rat}}^{-0.25}} \times t_{0.5} \text{ (cow or rat)} \quad (3)$$

The live weight of the roe deer in this study averaged 19.7 kg (SD 2.1 kg, Table 1). The half-life for carbon in blood plasma (and therefore in the hoof) for a cow (body mass: 650 kg) is 29 day (adopted from [26]) and the half-life for rat (body mass 0.4 kg) is 5 day (adopted from [27]). The calculated mean half-life for roe deer then becomes 12.3 day (SD 0.3 day, Table 1). This period was added to the estimated date of maize harvest to calculate the time when half of the maize signal in the hoof had disappeared (inflection point).

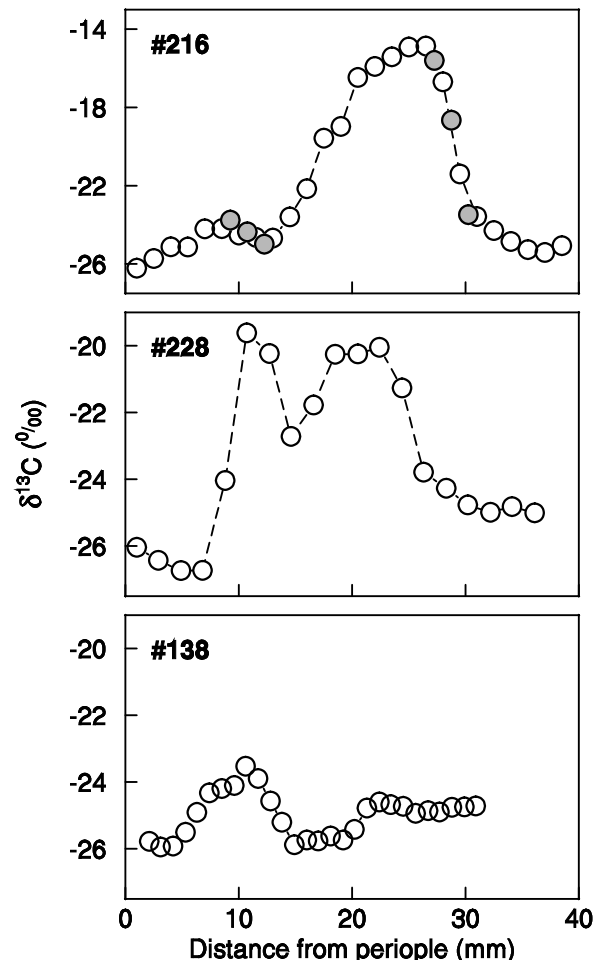


Figure 2. Longitudinal variation of $\delta^{13}\text{C}$ along the anterior edge of the hoof sampled from animal #216, #228, and #138. Note that y scaling differs for #216. Grey filled circles show data from a second sampling.

The $\delta^{13}\text{C}$ value of the half-life point on the hoof was determined as:

$$\delta^{13}\text{C}_{0.5} = \left(\delta^{13}\text{C}_{\text{with maize}} - \delta^{13}\text{C}_{\text{w/o maize}} \right) \times e^{-kt_{0.5}} + \delta^{13}\text{C}_{\text{w/o maize}} \quad (4)$$

where $\delta^{13}\text{C}_{\text{with maize}}$ is the last value of the maize peak and $\delta^{13}\text{C}_{\text{w/o maize}}$ is the first $\delta^{13}\text{C}$ value after the maize signal has disappeared in the hoof. The turnover constant k was retrieved as:

$$k = \frac{\ln(2)}{t_{0.5} \text{ (roe deer)}} \quad (5)$$

The position of the sample closest to the calculated $\delta^{13}\text{C}_{0.5}$ was determined as inflection point for each hoof. If $\delta^{13}\text{C}_{0.5}$ was between the values of two samples, the inflection point was set in the middle between those samples.

The differences in the time between when the animal was hunted and the date of C4-inflection will be called time since harvest (TSH) and the distance from the inflection point to the periople, “DIP”. Having the DIP and the corresponding TSH, we were able to calculate the growth rate (GR) of the examined hooves as:

$$GR = \frac{DIP}{TSH} \frac{[mm]}{[d]} \quad (6)$$

Total length of the hooves differed between 30 mm and 48 mm (Table 1). Using relative values instead of absolute distances could improve the relation of distance to time for all observed animals. Therefore, we also calculated a relative GR where DIP is expressed relative to total hoof length (which assumes that long hooves grow faster). To examine whether the correlation coefficients calculated with relative or with absolute GR were superior, we applied the Hotelling test of correlated sample sets [29].

The hoof GR were then used to reconstruct the annual variation of $\delta^{13}C$ in hoof keratin. To this end, the middle position of each sample was converted to a date by using the average GR. For comparison, either the average absolute or relative GR were used. Then one half-life was subtracted to account for body turnover and to yield the day when the feed was ingested. For the short distances between adjoining measurements along one hoof, we interpolated linearly. Values of $\delta^{13}C$ were averaged for all animals for which data were available for a certain day. This yielded a mean annual course of $\delta^{13}C$ in keratin with daily resolution. This annual course was compared to the average development of maize cover in the region taken from [22].

3. Results

3.1. Measured $\delta^{15}N$ and $\delta^{13}C$

The hooves had a mean $\delta^{15}N$ of 7.3‰ (SD 2.0‰), indicating that the animals predominantly fed on agricultural ground. However, the range of values among the animals was large (varying between 3.4 and 9.0‰).

The animals differed significantly in mean hoof $\delta^{13}C$ (ranging from −27.0‰ (SD 0.8‰) to −22.0‰ (SD 3.8‰), $p < 0.001$). With increasing mean $\delta^{13}C$ of a hoof, SD increased, indicating that those animals that consumed more maize had a more pronounced seasonal variation. This result was corroborated by $\delta^{15}N$, as hooves showing high mean $\delta^{15}N$ values (indicating pronounced feed on arable land) also had a high SD in $\delta^{13}C$ (Figure S1A,B in the supplementary information).

Although the rumen content provides only a snapshot while 1 mm of hoof including turnover time may reflect at least some days, $\delta^{13}C$ of the rumen content and $\delta^{13}C$ of the first increment of the hooves arranged close to the 1:1 line, when accounting for the diet-keratin shift. This confirmed the general assumption, that $\delta^{13}C$ in diet can be retrieved from hoof keratin (Figure S2).

3.2. Variation of $\delta^{13}C$ along the Hooves

There was a clear peak in $\delta^{13}C$ at a certain distance to the periople for 12 of the observed animals that was well above their $\delta^{13}C$ baseline (e.g., #216 in Figure 2). Correspondingly, these 12 animals also showed a relatively high SD in their $\delta^{13}C$ values and high mean $\delta^{15}N$ values (Table S1). Three animals (#118, #138 and #212; for #138 see Figure 2) showed only little variation in their $\delta^{13}C$ values (Table S1), which was corroborated by a small mean value in $\delta^{15}N$ (at least for #138 and #212), indicating that these animals fed less on arable land. However, an inflection point could still be detected and the animals were not excluded from the further calculation. For two animals (#134 and #228), the variation of $\delta^{13}C$ differed from the expectation, as they showed a second peak in $\delta^{13}C$ (#228 in Figure 2). Therefore, we excluded these two animals in a first approach to calculate the GR but included them later on.

3.3. Calculated Growth Rates

Following the assumptions that the last maize was harvested by DOY 306 and that half-life was 12 d (Equation 3), the date of the inflection point was 14 November (DOY 318). TSH varied between

97 day and 185 day and the DIP between 8.8 mm and 27.2 mm. The regression between TSH and DIP yielded a mean hoof GR of 0.130 mm/day (95% interval of confidence [CI]: 0.010 mm/day, $R^2 = 0.983$, $p < 0.001$, Figure 3A), if two animals (#134 and #228) that had a second peak were excluded. Including the two animals with two pronounced peaks yielded a similar result if the second, more distant peak was used for the regression, while the first peak clearly was out of focus. Hoof GR then was 0.122 mm/day (95% CI: 0.014 mm/day, $R^2 = 0.955$, $p < 0.001$), implying that a sampling width of 1 mm along the anterior edge of the hoof captured about 8 day of hoof growth. The first peak of the animals #134 and #228 came closer to the regression if it was assumed that it resulted from winter feeding by hunters using maize and that this winter feeding ended in early February (including turnover, Figure 3B).

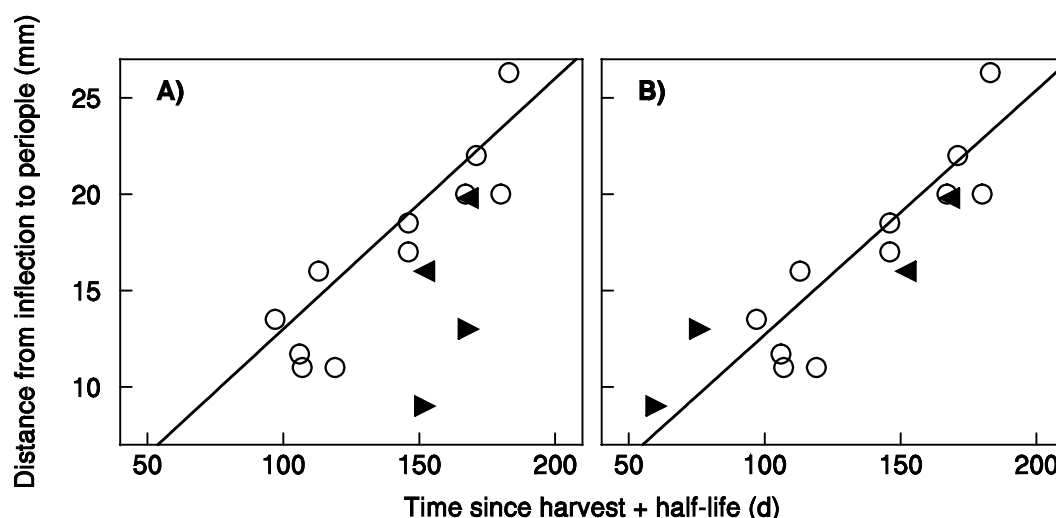


Figure 3. Distance between the inflection point and the periople depending on the time since the maize harvest plus one half-life at the date of hunting. In (A) only the animals with one clear peak (open circles) were used to calculate mean growth rate (0.130 mm/day; line); In (B) also the more distant peaks (left-tipped triangles) of the two animals with two peaks were used to calculate growth rate (0.122 mm/day). The peaks closer to the periople (right-tipped triangles) were assumed to result from winter feed provision. They are plotted at a position which would result if winter feed provision ended in early February, but were not included in the calculation of mean growth rate.

Finally, with respect to the different length of the hooves, we calculated a relative GR. The regression improved (R^2 increased from 0.955 to 0.982) and the Hotelling test proved that using the relative distance performed significantly better ($p < 0.05$). Hooves grew 0.365% (95% CI: 0.026%) of their total length each day. From this follows that roe deer hooves store the information of 274 day (nine months). Differences in hoof GR were apparently not caused by different live weights of the animals as there was no correlation between GR and live weight either for absolute ($R^2 = 0.009$) or relative ($R^2 = 0.102$) values.

Sampling positions could be converted to dates by applying the average GR and considering the date of hunting. Given a storage period of nine months and a period of three months during which the animals were hunted, a full year was covered. Keratin $\delta^{13}\text{C}$ started at about -26‰ at the time when maize was sown (on average 20 April) and then increased in parallel to the increasing maize cover (Figure 4A). This demonstrated that the hoof GR, which were determined between 14 November and the day of hunting, could be successfully applied until early May and thus appear to be valid even when extrapolated over six months. This extrapolation provided another line of evidence that relative GR were superior to absolute GR. While the seasonal variation in $\delta^{13}\text{C}$ followed the expectation from the typical time course of maize cover when a relative GR was used (Figure 4A), an unlikely peak

occurred in May/June when using the absolute GR (Figure 4B). This peak cannot be explained by the maize cover that is low during this month. Using relative GR placed the high values leading to this peak (reasonably) into June. After maize harvest, $\delta^{13}\text{C}$ still was high but gradually decreased as body pools turned over and returned to -26‰ mid of January. Similarly, there was a delay mainly in July between maize cover and increasing $\delta^{13}\text{C}$ in hoof keratin, which again can be attributed to body turnover.

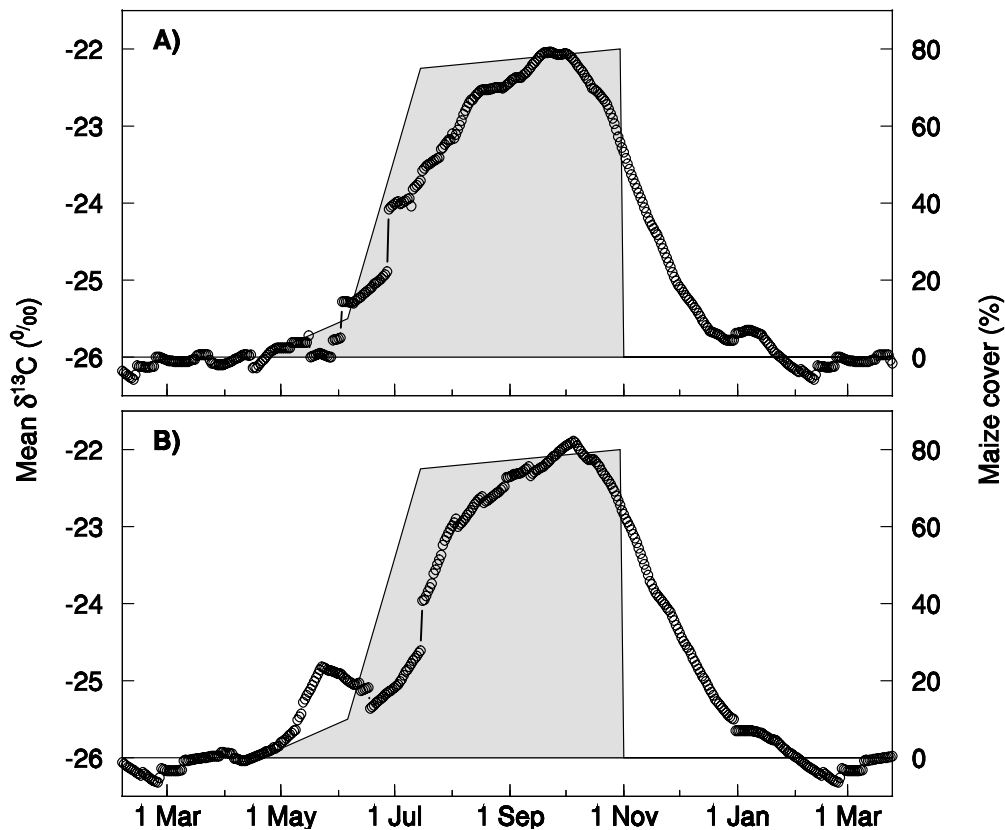


Figure 4. Daily mean $\delta^{13}\text{C}$ in hoof keratin (circles) versus time averaged over 17 animals hunted between mid-February and mid-May. Mean SE for 17 animals is 0.44‰ . Average development of maize cover (shaded area) within the research region was taken from [22]. In (A) time was derived from the distance to the periople, a relative growth rate of $0.365\%/ \text{day}$ and the date of hunting; a half-life of 12 day was subtracted. In (B) time was derived from the distance to the periople, an absolute growth rate of $0.122 \text{ mm}/\text{day}$ and the date of hunting; a half-life of 12 day was subtracted.

4. Discussion

Most animals showed pronounced feeding on maize, which caused up to 85% of the theoretical change in $\delta^{13}\text{C}$ when shifting from a pure C3 to a pure C4 diet. This agrees with the observation that roe deer select habitats of high feed availability and low visibility [30]. Our estimated GR of $0.122 \text{ mm}/\text{day}$ (95% CI: $0.014 \text{ mm}/\text{day}$) for roe deer hooves is within the range of known hoof/claw GR of other ungulates, such as cows and pigs. Harrison et al. [14] reviewed cattle (*B. taurus*) hoof GR and reported a mean of $0.21 \text{ mm}/\text{day}$ (SD $0.08 \text{ mm}/\text{day}$). Growth rates and variation were higher than for roe deer in our study. According to [14], the variation mainly resulted from differences in keeping conditions. High GR were found for cows in confinement, where the floor is hard and hooves therefore wear down fast [15]. Excluding the hoof GR of cattle kept in confinement from [14], leads to a lower GR of $0.16 \text{ mm}/\text{day}$ (SD $0.03 \text{ mm}/\text{day}$).

As relative GR were superior to absolute GR in our study, we transformed the absolute GR from [14] to relative rates using a total claw length of 72 mm (adopted from [31]), yielding a mean relative GR of 0.29%/day (SD 0.11%/day).

For pig (*Sus domesticus* L.), [20] found a mean claw horn GR of 0.18 mm/day (SD 0.05 mm/day). For the cranial position of the outer claws of the front limb (representing a similar location to where we sampled the roe deer hooves), the GR was 0.11 mm/day (SD 0.01 mm/day), while hind limbs grew faster by 0.10 mm/day (95% CI: 0.3 mm/day, recalculated from [20]). Inner and outer claws did not differ significantly, but GR in caudal position was slightly higher (by 0.02 mm/day, 95% CI: 0.01 mm/day) than in cranial position. Similarly, an increase in GR from the anterior to the posterior region was found for the bovine hoof [14]. This indicates that our GR may only be applicable for the cranial position of front hooves. When calculating relative GR from the data in [20], the differences between positions did not disappear but increased, indicating that relative GR are at least not suitable for eliminating positional effects. The relative GR for the cranial position of the outer claws of the pig's front limb was 0.30%/day (SD 0.05%/day [20]).

Hoof GR in general appear to be considerably slower than hair GR. For cattle, where hair and hoof GR are known, hair GR usually range between 0.6 and 1.0 mm/day [12,13,32], which is about two to four times higher than hoof GR. The slower GR of hooves, therefore, is advantageous considering the short length of the hooves but restricts the temporal resolution.

The variation in calculated turnover times due to different live weights of the roe deer was very low (SD of 0.3 day). As this small turnover variation hardly had an influence on the calculated GR, it could be neglected in our study as a possible source of calculation uncertainties. A larger uncertainty resulted from the assumption when maize harvest ended or when roe deer stopped feeding on maize. We assumed that all roe deer would feed on maize until end of maize harvest. However, the time of harvest varies from year to year depending on weather, location, variety of maize or designated use and roe deer could stop feeding on maize before harvest. If we assume an uncertainty of 14 day in TSH, while TSH varied between 97 and 185 day, this will cause an uncertainty in hoof GR of about 10% (equal to our 95% CI). Hence, our results seem to be quite robust regarding uncertainties in turnover time and date of maize disappearance. Nevertheless, more precise data on regional maize harvest could be used in future studies to improve this method of hoof GR determination.

Growth and wear rate could differ throughout the year instead of being constant. Hahn et al. [33] reported that bovine hooves grow faster in warmer months. Similarly, GR of cattle hair also were slightly higher (12%) in summer than in winter [16]. Conversely, Harrison et al. [15] found highest GR of bovine hooves in winter and explained these findings by differences in diet during the year. As wild animals change their behavior concerning feed, living environment or energy metabolism during the year [34–36], there are most likely also seasonally differing GR. However, varying GR by $\pm 10\%$ (yielding a 20% variation in total) hardly changed Figure 4. The reason is that the mean length of 35 mm and a mean GR of 0.122 mm/day suggest a total record of 292 day. Even if half of this record would have a 10% lower (respectively higher) GR, this would only lead to a maximum displacement of the last millimeter by 13 day and less for the others. Still, our estimate gives the average GR over the whole year and cannot take into account seasonal variations.

Growth rate likely varies also during lifetime. This is especially pronounced in the case of horns (e.g., [11]) but also GR of cattle tail hair decreased with age by 7%/year [16]. Assuming a similar decrease in hoof GR and a roe deer lifespan of 10 year [19] leads to a maximum variation of $\pm 35\%$ around our mean GR. The age effect may thus be stronger than the seasonal effect because it also affects the entire hoof and not only the part that grew during a certain season. Age may thus deserve future attention.

Our analysis provided two significant but weak indications that relative GR were superior to absolute GR when comparing roe deer with different hoof lengths. This finding does not apply generally because for pigs it could not explain the differences among hooves of one animal. Further evidence is thus needed whether and in which cases relative GR are preferable.

5. Conclusions

We were able to estimate growth rates of roe deer hooves with only one known date (date of death) by calculating a second date from the decrease in $\delta^{13}\text{C}$ due to the change in feed, which is provoked by the harvest of maize. The method proved to be quite robust against uncertainties in the calculation like the exact date of maize harvest or variation in turnover-times due to different live weights. The method may be applied to determine growth rates of other incrementally growing tissues in wild animals. Further, the growth rate determined here fosters dating the isotopic information in roe deer hooves also in cases where the sudden change induced by the harvest of maize is missing. This will facilitate studies on nutrition ecology of roe deer.

Supplementary Materials: The following are available online at <http://www.mdpi.com/1999-4907/8/12/462/s1>, Figure S1: Relation between the seasonal variation of $\delta^{13}\text{C}$ and the mean $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$ in hoof keratin, Figure S2: Comparison of $\delta^{13}\text{C}$ of the rumen content and $\delta^{13}\text{C}$ of the youngest millimeter of the hoof wall close to the periople, Table S1: Statistical parameters of isotope ratios in hooves of 17 analyzed roe deer. The data analyzed in this study are archived at ResearchGate [37].

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