Aging through growth marks in teeth of Spanish red deer

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Abstract We carried out a study on age determination for Spanish red deer (*Cervus elaphus hispanicus*) from growth marks in dental cementum. We investigated the age at which the first growth layer and first rest line appear in cementum of incisors (I_1), molars (M_1), and canines (C), and explained how to interpret these marks to determine age. We evaluated accuracy and precision of aging with rest lines of different tooth types. Age was estimated using a standardized counting method and a linear regression analysis, both based on rest-line number in permanent teeth of red deer aged between 4 and 44 months. The first growth layer occurred after dental eruption in all teeth studied. The first rest line appeared at 6 months in molars (M_1) and at 15 months in incisors (I_1). The second rest line appeared at 18 months in molars and incisors. Each consecutive rest line represented one year more in animal age. Subsequent analysis showed that canines do not give an accurate estimation of the real age. Molars and incisors did not yield the same values for age estimation. Molars gave the best results, aging 75% of animals correctly, while only 49% were aged correctly using incisors. Nevertheless, within a one-year confidence interval, 99% of the animals were aged correctly using molars, versus 86% using incisors. We suggest using the first lower molar for deer aging in Sierra Morena. Use of regression should be preferred to the standardized counting method for age determination when using incisors.

Key words aging, *Cervus elaphus hispanicus*, dental cementum, first rest line, southeastern Spain, Spanish red deer

Since the early twentieth century, skeletochronology has been used to determine age in vertebrates. This technique is based on identifying growth marks formed in hard tissues (tooth and bone). Dental cementum is most frequently used for age determination of land mammals (Fancy 1980). Today, this technique is considered more accurate than others and is preferred for ascertaining whether various physical and population parameters change significantly with animal age (Hamlin et al. 2000). However, some basic assumptions on the technique need to be documented for correct use of dental cementum to age wildlife species. Grue and Jensen (1979) suggested verifying that growth layers are deposited regularly and identifying the season of formation of each mark, along with age of forming the first layer in each tooth type. The sequence of appearance of growth layers in dental cementum of red deer (*Cervus elaphus hispanicus*) from southeastern Spain is regular and seasonal (Azorit et al. 2002*d*). Two growth marks are formed per year—a wide mark during spring–summer called the growth layer and a thin rest line resulting from reduced growth during autumn–winter (Azorit et al. 2002*d*). Variation among teeth and species with respect to season of

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formation stresses the need for each species and investigation to be coupled with an evaluation (Morris 1972, 1978). The counting method and its interpretation must be standardized to avoid subjective errors of aging (Matson et al. 1993, Landon et al. 1998).

Previous studies have confirmed the regularity of deposition of growth marks and identified the seasons of their formation (Azorit et al. 2002*d*). In addition, techniques, methods, and most appropriate parts of teeth to be used in southeastern Spain were also investigated (Azorit et al. 2002*b*,*c*). In the present work, we investigated the age at which the first growth layer and first rest line appeared in incisors (I_1), molars (M_1), and canines (C) of Spanish red deer. We described a standardized counting method to deduce age by counting dental growth marks, along with use of a linear regression for age prediction from growth mark number. To evaluate both procedures, we studied the magnitude and direction of bias, and the frequency of errors associated with age estimation of red deer in southeastern Spain.

Materials and methods

We obtained teeth from deer killed from 1993 to 1997 in sport-hunting seasons and management culls in Sierra Morena, southeastern Spain (Figure 1). In Sierra Morena deer, the first lower molar (M_1) erupts at 6 months, the first incisor (I_1) at 15 months, and the canine (C) at 16 to 18 months of age. On the other hand, the third cuspid of the third lower molar (M_3) finishes its eruption at 42–44 months (Azorit et al. 2002*a*), which allows aging animals up to this age. To verify whether rest-lines formation takes place before or after dental eruption, we extracted some partially erupted molars, incisors, and canines from deer of different ages: molars from deer <6 months, incisors from deer <15 months, and canines from deer ≈16–18 months of age. We analyzed the 2 first incisors (I_1) or the 2 first lower molars (M_1) from several deer when they presented different degrees of eruption (1 incisor or 1 molar fully erupted while the other was not fully emerged and lacked complete dental occlusion). We analyzed 141 teeth: 55 first lower molars (M_1) from 51 deer, 64 incisors (I_1) and 22 canines (C) from 61 deer. The ages of animals ranged from 4–44 months and were established by estimating the interval from known date of death and birth season, as described in similar studies (Attwell 1980, Vigal and Machordom 1985, Genov et al. 1992, Pérez-Barbería and Mutubería 1996, García-Perea and Baquero 1999). Most births occur from May–June in Sierra Morena (Azorit 1999).

We prepared histological sections from permanent first lower molars (M_1), first incisors (I_1), and canines (C). We decalcified teeth using a commercial preparation containing 14% hydrochloric acid and polyvinylpyrrolidone (TBD-1® Shandon, Cheshire, England). We embedded teeth in paraffin, sagittally cut midlines with a conventional microtome, and stained them with Haematoxylin-Eosin, yielding thin histological sections 4–5 μm thick (Azorit et al. 2002*c*). We observed sections through a light-transmitting microscope at 10X or 40X magnification, which performed better than a polarized transmitting light microscope (Azorit et al. 2002*b*). With this method, rest lines appeared as narrow, dark-stained, eosinophilic lines between wider growth layers. We made observations using a video

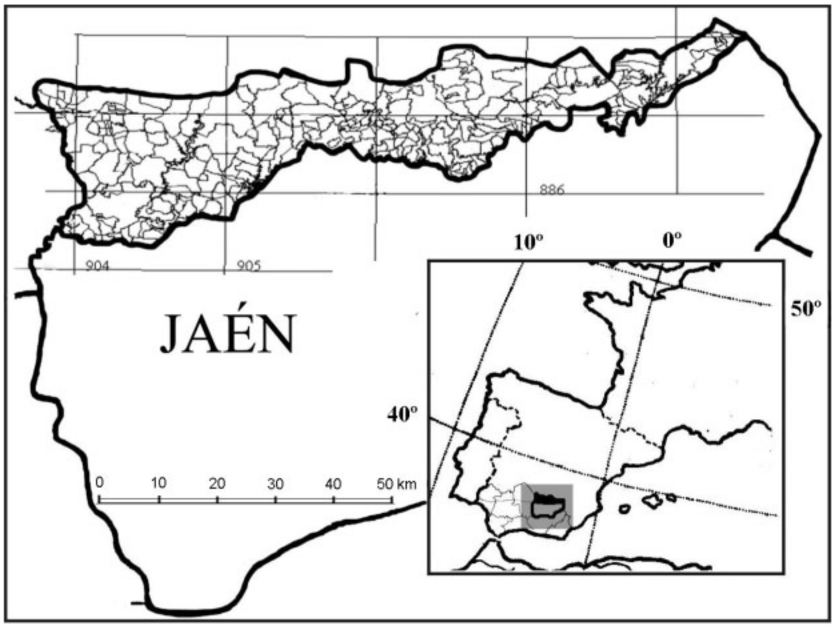

Figure 1. Map and geographic coordinates of the study area, Sierra Morena, Spain.

camera connected to a computer, allowing simultaneous observation by several people, to reduce subjectivity errors in the aging process (Matson et al. 1993, Landon et al. 1998).

First, we standardized a method of counting growth marks to determine age. Then we estimated age in 2 ways: using the standardized counting method (SCM) or using the linear regression of animal actual age in months against number of growth marks in a given tooth (canine, first incisor, or first lower molar). An animal was considered correctly aged when the absolute difference between known and predicted values was <6 months (Moore et al. 1995). An error of 1 year was assumed when the difference was 6 to 18 months, an error of 2 years was assumed when this difference was 18 to 30 months, and so on.

Evaluation consisted of comparing real age with age estimated by each procedure. Correlation between real and predicted age and evaluation of the global error through mean square error and bias were compared between both procedures. All statistical analyses were done using SAS (SAS 1992).

Results

Standardized counting method for age determination

We tried to outline the specific SCM for aging deer through dental growth marks, using molars, incisors, and canines (Figure 2).

Molar (M_1)

In deer <6 months, growth of the cementum layer started with dental eruption. The first growth layer was thinner than subsequent layers (Figure 3A). The first thin, dark rest line appeared at 6 months at the earliest and coincided with dental occlusion (when the tooth is fully functional and adjoins its opposite). This corresponded to the period of less growth in dental cementum (November–January). Thus, the first growth layer in molar cementum developed during the spring–summer growing season and the first rest line during the deer's first winter when the animal was 6 months old. The second growth layer was deposited during the next spring–summer. The second rest line corresponded to growth slackening during the next winter; thus, the second rest line corresponded to an age of 18 months, the third to 30 months of age, and the process repeats itself throughout the animal's life.

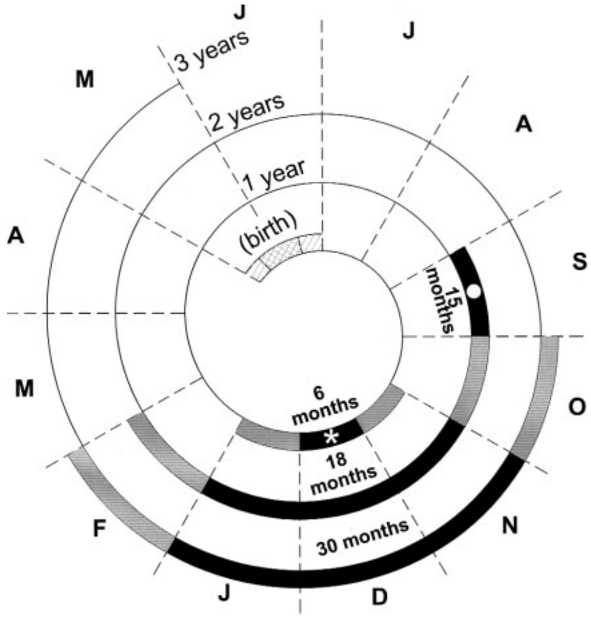

Figure 2. Standardized counting method for aging through rest lines in molars (M_1) and incisors (I_1) of Spanish red deer. Starting at 18 months, subsequent rest lines form periodically each year both in molars and incisors. Wheel represents a year and letters represent months. Solid bars = period for cementum low growth and rest lines development. Black solid bars = period of time when most rest lines take place. Asterisk = moment of appearance of first dark line in molars (M_1). White circle = moment of appearance of first dark line in incisors (I_1).

Incisors (I_1)

The first growth layer is deposited just distal to Tomes' granular layer of dentine. Both are separated by a thin, dark line called dentine-cementum junction, which should not be mistaken for the first rest line in dental cementum. The first growth layer is formed during dental eruption. Permanent incisors from animals under 15 months of age showed only 1 growth layer varying in thickness. At 15 months the first rest line was formed, coinciding with the end of dental eruption and occlusion (Figure 3B). We will refer to this as the "occlusal line." As in other artiodactyla, this line was visible even before the incisor root was completely closed (Lieberman 1994). Up to 18 months of age, there appeared a hyaline cementum layer, the first rest line or occlusal line, and a subsequent growth layer. Starting at 19 months, the second rest line appeared, coinciding with slow cementum growth in November–January. Therefore, if an animal is born in May–June, since the incisor erupts at 15 months of age, 2 rest lines appear in the incisor during the second year of life. At this time an acellular cementum layer varying in thickness can be

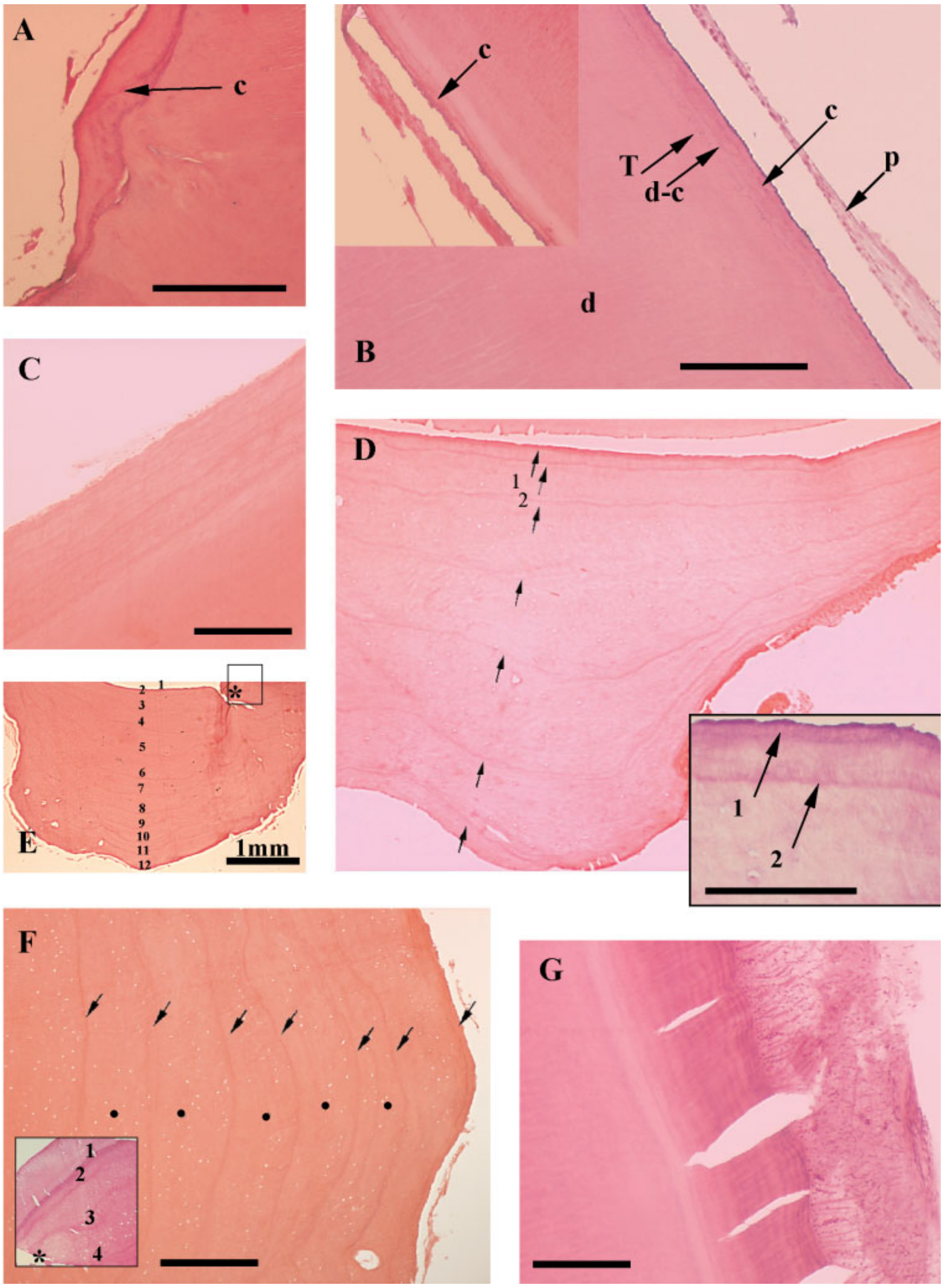

Figure 3. Histological thin sections (4–5 µm) obtained with a sample embedded in paraffin and stained with hematoxylin-eosin and cut using a conventional microtome. (A) First lower molar (M_1) of a 15-month-old deer killed in September. c = first rest line (magnification: 10X, scale bar = 150 µm). (B) First rest line in cementum of the first incisor (I_1) of the same deer. d = dentine, T = Tomes' granular layer, d–c = dentine–cementum junction, c = first rest line, p = periodontal membrane (magnification: 10X, scale bar = 100 µm). Insert is a histological thin section (4–5 µm) of the first incisor (I_1) of another 15-month-old deer. (C) A canine showing the growth marks in cementum (magnification: 10X, scale bar = 150 µm). (D) First molar (M_1) of a deer killed in February at an estimated age of 78–80 months. Insert shows details of the first rest line in this same molar (magnification: 40X, scale bar = 50µm). (E) First lower molar (M_1) of a deer killed in January. Twelve annual rest lines are observed corresponding to an age of 11.5 years. The last rest line is currently being formed at the periphery of the root and is not clearly visible in this photograph, making necessary its observation at 10X magnification minimum. (F) Molar of the same deer as in E. The last growth layer is very thin, and the last rest line is at the edge of dental cementum. Asterisk indicates secondary lines. Insert shows detail of the first rest line in this same molar (magnification: 10X, scale bar = 150µm). (G) An incisor of the same deer as in E and F.

observed between the first rest line and the second. This first rest line (the occlusal line) is caused by dental occlusion, while the second line is due to reduced growth during winter (when the deer is 18 months old). The third rest line is formed during the next winter, when the deer is 2.5 years old, and so on throughout life.

Table 1. Parameters (estimate ± SE) and determination coefficients (R^2) of the linear regression fit between actual age and growth mark number in incisors, molars, and canines from red deer in Spain, 1993–1997.

Tooth	Intercept	Slope	R^2
Incisor I_1	12.66 ± 1.94	8.16 ± 0.79	0.66
Molar M_1	6.46 ± 2.40	8.90 ± 0.90	0.70
Both I_1 and M_1	8.00 ± 2.55	2.73 ± 1.92	0.70
	6.04 ± 2.03		
Cementum of canine C_1	28.68 ± 3.02	1.80 ± 1.45	0.03

Canines (C)

We observed periodic growth marks in all canines analyzed. It was not possible to determine the exact moment of first rest-line formation because of the high variability among samples. We observed a growth layer in deer <16 months but observed no rest line. There were canines with closed roots not showing rest lines, while others, with roots still open, showed several growth layers with associated rest lines. Deer 18–20 months old showed 2 rest lines in molars and incisors, while canines showed up to 3 lines. Even if we observed growth marks in cementum and dentine (Figure 3C), the numbers of counted rest lines did not agree between both tissues or with the other teeth. This limits use of canines for reliable deer aging.

Age determination

Deer age in years can be determined by counting growth layers or rest lines in molars (Figures 3D, E, F) and incisors (Figure 3G). However, rest lines proved easier to count. The first rest line in molars corresponded to an age of 6 months, while in incisors it corresponded to 15 months. The second rest line corresponded to an age of 18 months in molars and incisors. Each successive rest line meant one year more. Hence, age in years of a given deer was equal to counted rest lines minus 0.5 years. If age in years and month of death of a given individual are known, age in months can be calculated assuming a known birth month (usually May–June in southeastern Spain). However, the number of months between the previous December (date of formation of the last rest line) and the month of death should be taken into account. But when month of death is unknown, it becomes very difficult to deduce animal age in months, especially in animals killed between February and October. A difference of ≤6 months can exist among animals all showing the same number of rest lines because rest lines take place after September, mostly between November and January (Azorit et al. 2002*d*). In these cases analysis of the last growth layer and its width can be useful for fine-tuning age determination. For example, a deer dead in October will show a wide last growth layer about to develop the rest line, while a deer dead in January or February should show a thin last growth layer still growing for up to 9 months or more until the next rest line developed (Figure 3F). In general, this is easier to observe in molars, where growth layers in cellular cementum are thicker than in acellular cementum of incisors.

Regression of age against growth mark number in molar, incisor, and canine

Canines did not show any relationship between rest-line number and actual age as indicated by R^2 values (Table 1). In other teeth, R^2 of linear fit between rest-line number and actual age was higher for molars than incisors and did not increase when both teeth were included in the same fit. On the other hand, R^2 was greater when males and females were separated (Table 2). The best fit was obtained in molars, and the corresponding linear equations were:

Age = 5.20 + 9.23 × (rest-line number), for males

and

Table 2. Parameters (estimate ± SE) and determination coefficients (R^2) of the linear regression fit between actual age and growth mark number in incisors and molars, from male and female red deer in Spain, 1993–1997.

	Females			Males		
	Intercept	Slope	R^2	Intercept	Slope	R^2
Incisor(I_1)	14.8 ± 2.53	7.55 ± 1.14	0.68	9.02 ± 3.06	9.92 ± 1.20	0.71
Molar (M_1)	5.20 ± 3.15	9.23 ± 1.21	0.76	4.41 ± 3.76	10.30 ± 1.47	0.73

Age = 4.41 + 10.30 × (rest-line number), for females.

Evaluation of procedures

Correlation between real and estimated age from molars was higher than from incisors (Table 3). Furthermore, when using incisors, age estimated with regression was more closely correlated to actual age than using the SCM. Mean square error (MSE) was higher in females than in males and higher using incisors than molars. Likewise, bias was higher in females and more often negative than in males (Table 3). About 75% of deer were aged correctly using molars, while only 49% were aged correctly using incisors. However, within a 1-year interval, about 99 and 86% of deer were correctly aged using molars and incisors, respectively. The most frequent errors were underestimations, mostly <1 year, but when using incisors the errors were ≤3 years, and more overestimations occurred (Table 4).

Table 3. Correlation coefficients between real and estimated age values, mean square error and bias, using linear regression or standardized counting method (SCM) in male and female red deer in Spain, 1993–1997.

	Procedure [a]	Correlation [b]	Mean square error	Bias (months)
Males	REGINC	0.81***	964.93	+1.10
	REGMOL	0.89***	603.05	+0.97
	SCMINC	0.67***	2,244.00	–4.50
	SCMMOL	0.89***	546.50	0.00
Females	REGINC	0.81***	1,457.00	–1.06
	REGMOL	0.93***	703.68	–0.32
	SCMINC	0.70***	3,360.00	–6.40
	SCMMOL	0.94***	1,037.00	–4.50

[a] Procedure used for age estimation: REGINC = age estimated by regression in incisor; REGMOL = age estimated by regression in molar; SCMINC = age estimated by standardized counting method in incisor; SCMMOL = age estimated by standardized counting method in molar.

[b] *** = $p < 0.001$.

Discussion

Previous studies on caribou (*Rangifer tarandus*) reported that the first rest line was formed before dental eruption (Reimers and Nordby 1968, Miller 1974). Nevertheless, we examined sections from teeth not fully erupted or still inside the gum and found no rest line prior to eruption. Our results coincided with studies on white-tailed deer (*Odocoileus virginianus*) by Gilbert (1966), Boozer (1969), and Thomas and Bandy (1975), who also examined histological preparations of decalcified teeth embedded in paraffin. However, our results differed from those of Mitchell (1967) or Douglas (1970), who used undecalcified thin-sections of molars (M_1) from red deer (*C. elaphus*), or histological sections obtained with a freezing microtome (Ohtaishi 1978, 1980, Ohtaishi et al. 1990) from Sika deer (*C. nippon*) or white-lipped deer (*C. albirostris*). These authors found that the first rest line formed during the second winter in an animal's life, not during the first winter, as we found. This is why it was necessary to add 1 to the number of counted lines to obtain the correct age (Mitchell 1963, Klevezal and Kleinenberg 1967, Erickson and Seliger 1969). However, the general rule of adding 1 to the number of lines is not always adequate, as some authors noted in other species (Zapata et al. 1995). The first rest line can take place during the first year of life, as we observed in molars (M_1). It also can vary with type of tooth and its eruption. Therefore, the aging procedure must be assessed for each tooth type and each species, or even for different populations of the same species (Grue and Jensen 1979). The discrepancy in results could be due to differences in the techniques or to differences in the standardized counting methods used by different authors, as well as to seasonal nutritional fluctuations or geographical and physiological differences among cervid species.

Several authors reported difficulties during identification and counting of growth marks, and variability among observers, regions, and teeth (Klevezal and Kleinenberg 1967, Lockard 1972, Roseberry 1980, Jacobson and Reiner 1989, McCullough 1996). For reliable and detailed age interpretation, it is necessary to identify these cases and, as recommended by Rice (1980), distinguish irregular (supplementary, false, split, or composite) marks. But differences among counting methods or techniques used are likely to be more important sources of discrepancies. When observing sections thicker than 15 μm with light-reflecting

Table 4. Error frequency when using growth marks from incisors and molars to estimate age of red deer in Spain, 1993–1997.

Error type:	Underestimation				Overestimation		
Error in years:	–3	–2	–1	0	+1	+2	+3
Error frequency:							
Molars	0	1.1	21.7	75.0	2.2	0	0
Incisors	3.4	10.2	32.2	49.5	5.1	0	0

microscopy, it is more difficult to distinguish the first rest line, which can even go unnoticed (Azorit et al. 2002*b*). The first rest line detected by most authors using undecalcified thin-sections should correspond to the second rest line when using decalcified sections. For this reason Gasaway et al. (1978) reported that skeletochronology tends to over-age moose (*Alces alces*) by an average of 0.5 years and commented on the subjectivity of interpreting growth marks. There were cases in which section quality was very important to ensure a correct identification of the first rest line formed in cementum. For example, in some samples the 2 first growth layers were very thin and the rest lines were so close that it was difficult to distinguish them. In very old animals, with a high number of growth layers, the pressure exerted by external layers likely led to blurred first rest lines. Gasaway et al. (1978) observed that growth layers of moose deposited during the first 3 years were less distinct than those formed in later years.

In the case of deer from southeastern Spain, molars were more suitable for age interpretation because their rest lines as well as their accessory lines were easier to observe than incisors (Azorit et al. 2002*b*), being more adequate than the use of the cellular cementum of the interradicular pad of the molars (Figures 3D, E, F). Furthermore, we observed a delay in incisor replacement in some deer. Some permanent first incisors did not erupt at the usual age of 15 months, but appeared as late as 18 months (Azorit et al. 2002*a*). In these cases, during the second year, a single rest line was formed instead of 2, probably due to coincidence of dental occlusion with reduction of growth. This can lead to errors in age determination using incisors. The latter may vary in time of formation of the first rest line due to delays in dental replacement. They also require some previous experience to avoid rest-line confusion with the Tomes granular layer.

Precision obtained using skeletochronology for age estimation of red deer was different between teeth. It is generally accepted that the typical accuracy in aging with growth layers is near 70%, with at least 90% within 1 year of the correct age (Matson 1981). Using molars, the values were 75% and 99%, respectively. With incisors, however, these values were 49% and 86%, respectively. Differences between teeth were obviously caused by differences in number of layers counted in each type of tooth. This could be attributed to differences in clarity of layers, which was more often lower in incisors. In fact, 41% of all deer more than 1.5 years old showed more marks in molars; in about 3% they showed more than 2 layers. On the other hand, SCM interpretation was always easier using the first lower molar (M_1). Moreover, in molars it was easier to detect the first and the last rest lines, while in incisors the first marks seldom were identified. Teeth from older animals have shown an amalgamation of the first marks, which makes it difficult to count the lines, increasing the chance of error with increasing age. This suggests that our calculated error probably was underestimated, because animals used were ≤4 years.

Our study showed more frequent underestimation than overestimation errors, in agreement with Moore et al. (1995). The most common error was similar to that obtained by Müller-Using (1981). Nevertheless, we did not detect error >2 years using molars, while values of 3 years were obtained with incisors. Finally, female age estimated through regression gave smaller mean square error (MSE) and smaller bias irrespective of type of tooth used. In contrast, regression was more efficient (smaller MSE and bias) in estimating male age with incisors, while the SCM was slightly more efficient in molars. In general, use of molars was more reliable. However, bias was always higher and more often negative in females, indicating an age underestimation surpassing 6 months. Canines should not be used for age determination due to variability in time of formation of growth marks and lack of a relationship between formation of the first growth layer and dental occlusion.

Management implications

Although canines can be easily extracted (even when the animal is still alive), they were not useful for age determination in Spanish red deer. In contrast, molars were the hardest teeth to extract but the easiest to interpret and the most accurate. Incisors were more easily extracted from mandibles than molars. But they showed a higher variability in the time of formation of the first growth layer, causing errors in age estimation. Consequently, we suggest using the first lower molar for deer aging in Sierra Morena, to avoid age underestimation errors that can lead to overestimation of population growth rates. Further, it can lead to cohort misidentification and life-table misinterpretation, with the subsequent risk of erroneous management decisions, leading to serious negative effects on wildlife populations and environment (Roseberry 1980, Vincent et al. 1994). Regression likely is more efficient than the SCM for aging when using incisors. However, this should be confirmed by using animals >44 months old. Moreover, regression allows estimating the age of an animal without prior knowledge of its month of death. This is an advantage over the conventional method of age deduction from the SCM of growth marks.

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