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Age determination in yellow-pine chipmunks (*Tamias amoenus*): a comparison of eye lens masses and bone sections

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Abstract: Virtually all biological characteristics of organisms change with age, and thus, to assess the impact of these changes, accurate aging techniques are essential. However, many current methods are unable to accurately distinguish among adults of different ages. We determined the age of yellow-pine chipmunks (*Tamias amoenus*) from the Rocky Mountains of Alberta using eye lens masses, annuli from mandible sections, and annuli from femurs. Each of these methods was assessed against nine known-age animals and seven animals that had not been caught previously and were presumed to be juveniles. Eye lens masses could distinguish juveniles from adults but not adults of different ages. Mandibular sections were not practical in this species because of excessive tearing during sectioning. Femoral sections precisely predicted age. We found that the number of adhesion lines, minus one, accurately represented the ages of adults ranging from 1 to 5 years old. Femoral annuli have not previously been used to age mammals and our results suggest that they may be useful in aging other mammals, especially rodents.

Résumé : Parce que pratiquement toutes les caractéristiques biologiques des organismes changent avec l'âge, il est nécessaire de posséder des techniques précises de détermination de l'âge lorsqu'on veut évaluer l'impact de ces changements. Cependant, plusieurs des techniques d'usage courant ne permettent pas de distinguer les adultes appartenant à différents groupes d'âge. Nous avons déterminé l'âge de tamias amènes (*Tamias amoenus*) des Montagnes Rocheuses de l'Alberta au moyen de la masse du cristallin, ainsi que des anneaux de croissance sur des coupes de mandibules et de fémurs. Chacune des méthodes à été évaluée à l'aide de neuf animaux d'âge connu et de sept animaux capturés pour la première fois et considérés comme des jeunes. La masse du cristallin permet de distinguer les jeunes des adultes, mais pas les adultes des différents âges. Les nombreuses déchirures survenues pendant la préparation des coupes des mandibules nous ont empêchés de les utiliser chez cette espèce. Les coupes du fémur ont permis la détermination précise de l'âge. Le nombre de lignes de jonction, moins une, équivaut précisément à l'âge des adultes qui ont entre 1–5 ans. C'est la première fois que les anneaux de croissance du fémur servent à la détermination de l'âge chez un mammifère; nos résultats laissent croire que la méthode pourrait s'appliquer à d'autres espèces de mammifères et, en particulier, à des rongeurs.

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Introduction

Enormous amounts of detailed laboratory research has highlighted how age affects physiology (Pedersen et al. 2001; Hung et al. 2003), but it is unclear how these findings map onto animals living in the natural world (for a review of aging see Kirkwood and Austad 2000). Virtually all measures of life history, morphology, and physiology show age-dependent changes in fundamental rates (survival, reproduc-tion, and growth), in structure, and in function (Ricklefs and Finch 1995; Wickens 1998). To make sense of these age-dependent differences among animals, it is critical to age them accurately. Although this is simple with laboratory-reared animals, accurately determining the age of free-living animals can be a difficult or impossible task. Marking animals as juveniles and then collecting them at older ages is often not a viable option because it is extremely labour intensive, time consuming, and site specific. One alternative is

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to use morphological traits that change at a known rate with age. Two potential methods that are commonly used are eye lens mass and annual adhesion lines (annuli) in bones or teeth.

The lens of the mammalian eye continues to grow after birth, and thus, its dry mass can be used to estimate age. Since its description by Lord (1959) for use in aging cottontail rabbits, this technique has been used to determine age in many mammal species, being particularly successful in medium-sized mammals including rabbits, squirrels, and deer (for review and references see Morris 1972; Friend 1967; for more recent examples see Catling et al. 1991; Kauhala and Soveri 2001; Stockrahm et al. 1996). It consistently distinguishes between juveniles and adults within various species with near-perfect accuracy. However, resolution declines with age, as the mass of the eye lens tends to asymptote within 1 or 2 years, and thus, one cannot accurately determine the age of longer lived adults.

The number of adhesion lines in bone may be a direct measure of age. Mammalian bone grows by the deposition of layers of bony plates in the periosteal zone on the outer surface. These layers are separated by parallel adhesion lines representing a delay in the growth of bone thickness (Klevezal and Kleinenberg 1969b). This deposition tends to follow an annual pattern, with rapid growth occurring in summer and slow growth occurring in winter. Thus, it is possible to determine an individual's age in years by counting the number of adhesion lines. The bone used most frequently is the mandible, since it is easily accessed in a fresh specimen and dislocated from the skull relative to other bones. Counting lines in the mandible can give accurate estimations of age in some species of rodents, lagomorphs, and carnivores (Hamar and Prodanascu 1971; Millar and Zwickel 1972; Franson et al. 1974; Klevezal and Mina 1990). However, since the root of a rodent's incisor extends well into the mandible, the mandible walls act as part of the incisor support system. This compresses the bone laterally, which may lead to the formation of accessory adhesion lines in the bone that are indistinguishable from the annually deposited lines and result in overestimation of an individual's age (Klevezal and Kleinenberg 1969c).

Visible layers of dentin and cementum are present in mammalian teeth and are analogous to the adhesion lines in bone. These layers have been used most commonly to age carnivores by counting the annuli in incisors or canines (Linhart and Knowlton 1967; Allen 1974; Driscoll et al. 1985; Catling et al. 1991). Incisor layers have also been used to determine the age in some rodent species (Klevezal and Mina 1990; Trunova and Klevezal 1999; Klevezal 2002). However, age estimates are not as reliable because the incisors of rodents continuously grow and are worn down.

As an alternative to these methods, we report the first validation of the use of the femur annuli to age a mammal. Relative to the mandible, the long bones tend to have thicker, tougher ligaments and tendons connecting them to the muscles and skeleton that make them more difficult to remove. Examination of the annuli in long bones has been used to accurately age free-living amphibians and reptiles up to 21 years (Halliday and Verrell 1988; Castanet and Smirina 1990; Misawa and Matsui 1999) and has been used to estimate age in various sciurid species (Klebanova and Klevezal

1966, cited in Klevezal and Kleinenberg 1969a), although validation with adult mammals of known age has to our knowledge not yet been documented. We used sections of the femur to age yellow-pine chipmunks (*Tamias amoenus*), a relatively long-lived rodent, from wild, tagged populations in the front ranges of the Rocky Mountains in Alberta.

Yellow-pine chipmunks are small, hibernating, ground-dwelling members of the squirrel family (Sciuridae) that become sexually mature (adult) after their first winter of life and live up to 7 years (Sutton 1992). In the temperate climate of western North America, they remain in their burrow from late fall until early spring, waking approximately once each week to eat from a cache of food stored in their burrow (Sutton 1992; Geiser et al. 1997). As a result, we expected that the growth of chipmunks would slow or cease in the winter months, similar to the growth of other hibernating mammals. Adhesion lines should then appear in their bones at the rate of one per year. As part of a larger study examining aging and neurogenesis in the mammalian brain, we sought to find a technique that would allow us to determine the age of adult individuals and to utilize previously untrapped animals for such research. We predicted that femoral sections would be an accurate and reliable method to determine the age of free-living chipmunks.

Materials and methods

Yellow-pine chipmunks were collected from the Kananaskis Valley of Alberta, Canada, in early September 2001 on grids described previously by Schulte-Hostedde et al. (2001). From 1998 through 2000, yellow-pine chipmunks were tagged with passive integrated transponder (PIT) tags (Anitech Enterprises, Inc., Markham, Ont.), most on emergence from their burrow in their year of birth (Schulte-Hostedde et al. 2002). We therefore knew the precise or minimum ages of these tagged animals, allowing us to compare the results of different methods of age determination with each other as well as with the known ages of our subjects.

Animals were trapped in Longworth live traps baited with whole oats and sunflower seeds and provided with cotton for bedding. Animals were sexed, weighed (±1 g), anaesthetized with halothane, and sacrificed by a terminal bleed by cardiac puncture. Chipmunks were then scanned with a PIT scanner to detect the presence of an identifying tag. At the field laboratory, the eye lenses were removed and frozen at –20 °C, along with the bodies. The bodies were transported to the University of Toronto at Scarborough where they were kept frozen at –20 °C until dissection. Eye lenses from each animal were dried for 5 days at 80 °C, and the total eye lens mass (±0.1 mg) for each individual was determined as the sum of both dried eye lenses.

Upon dissection, the entire mandible and both femora were removed from each animal and fixed in formol–acetic–alcohol fixative for 24 h. The bones were then decalcified by submersion in rapid decalcifying solution (Apex Engineering Products Corporation, Plainfield, Ill.) for 24 h. A 3- to 5-mm section from midway along the length of the femur and another from the diastema of the mandible were removed for sectioning. These sections were dehydrated by submersion in increasing concentrations of *t*-butanol and then submerged in molten paraffin wax (TissuePrep2)

(Fisher Scientific, Nepean, Ont.) in a Tissuematon automated system (Fisher Scientific). The bones were then embedded in paraffin blocks and sectioned laterally with a microtome in 5- to 20-mm slices. The resulting slices were affixed on microscope slides, the excess wax was removed with Histoclear (National Diagnostics, Atlanta, Ga.), and the slices were stained with a hematoxylin–eosin stain. The slides were observed under a compound microscope at 100× and the thin, dark adhesion lines were counted by an observer blind to previous measures of age.

The lines in mandible sections were not as strongly defined as those present in the femoral sections. The decalcified mandibles were also technically difficult to section, with many of the sections tearing. The femoral sections were not prone to tearing. Since the mandibular sections were in such poor condition, only those lines found in the femoral sections were counted.

As an additional technique for classifying individuals as adults or juveniles, the gonads were examined. During dissection, the testes of males were removed and weighed. The uteri of females were removed and examined for implantation sites that appear as dark spots in the uterine wall where embryos had implanted. Since chipmunks do not breed until the spring after their birth, these measures allowed us to distinguish young of the year from adults.

Numerical results are means ± 1 SE. Statistical tests as specified in Zar (1984) were performed using the program StatView (Caldarola et al. 1998). We used the Tukey–Kramer multiple-comparison post-hoc test to examine the significance of main effects.

Results

Nine animals (six males and three females) with identifying PIT tags were captured. The ages of six of these were known with certainty because they had been captured in their year of birth (four were 1 year old and two were 2 years old). Of the rest, one was at least 3 years old (tagged as an adult in 1999) and two were at least 4 years old (tagged as adults in 1998).

Chipmunks known to be adults all had total eye lens masses over 13 mg, whereas total eye lens masses of seven trapped animals without tags (three males and four females) were less than 12 mg. Since smaller lens masses should be found in younger animals, we classified these untagged animals as juveniles. The relationship between eye lens mass and age quickly reached an asymptote at about 1 year of age and approximately 14 mg, with little possible distinction thereafter between adults of different ages (Fig. 1) (Tukey–Kramer post-hoc comparisons between adult ages, $P > 0.05$).

Body mass could not be used to distinguish between juveniles and adults. A statistical difference between adults (females 58 ± 1 g, males 54 ± 1 g) and juveniles (females 55 ± 1 g, males 50 ± 2 g) of the same sex may exist, but our low sample sizes provided insufficient statistical power to detect it (Tukey–Kramer, $P > 0.05$ for all pairwise comparisons).

Adhesion lines were clear in the femoral sections (Fig. 2). There was at least one dark band present in all animals, including juveniles. Once this first line was accounted for, the number of remaining lines corresponded exactly to the number of winters survived by six animals of known age. Al-

Fig. 1. Total dry eye lens masses of known-age and presumed juvenile yellow-pine chipmunks, *Tamias amoenus*. Data points for 1- and 2-year-olds are animals of precise known age, those for 3- and 4-year-olds are animals caught as adults in 1998 and 1997, respectively, and those for juveniles (0 years of age) are untagged animals whose lens masses were <12 mg. Numbers beside each set of data points represent the number of animals included in that set.
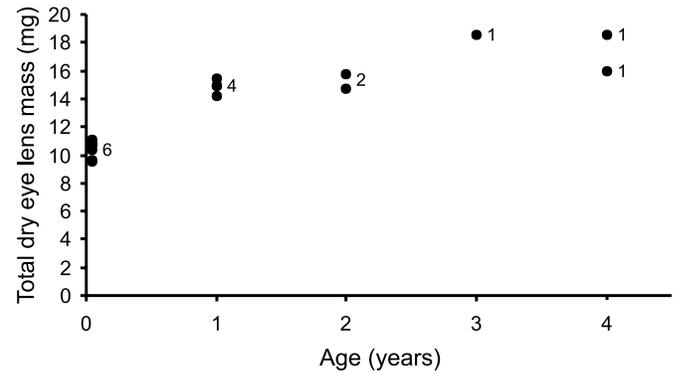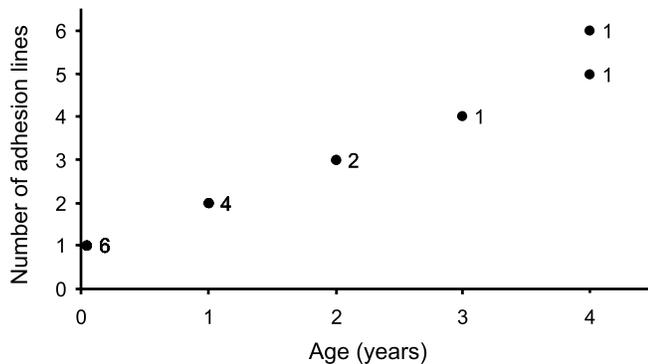

Fig. 2. Cross-section of femur from a 5-year-old yellow-pine chipmunk. Arrows indicate dark bands in the bone that indicate age in years; some are out of focus and appear faint. Note the apparent absence of a distinct mesosteal zone between the periosteal zone and the marrow cavity (MC). Despite the proximity of the dark bands to the marrow cavity, they corresponded exactly to the age of the individual in years. Scale bar = 100 μm.
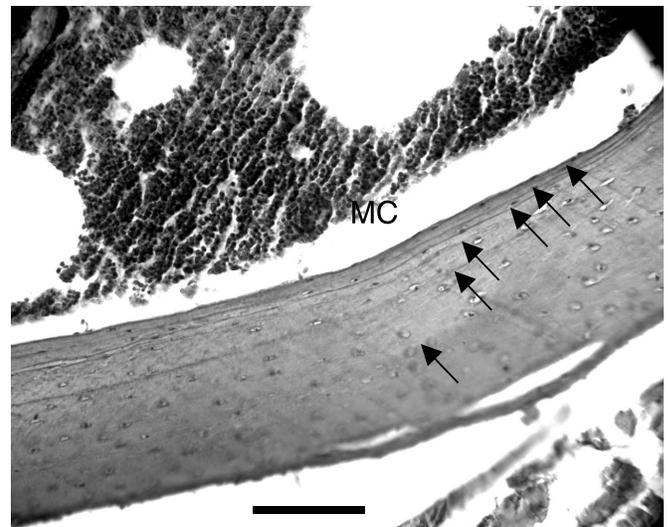
though we only knew the minimum, but not absolute, age of three of the animals examined, the number of dark lines present less one was equal to or greater than the minimum number of winters survived by these animals. The animal known to be at least 3 years old had four lines, for an estimated age of 3 years old. Another, known to be at least 4 years old, had five lines, for an estimated age of 4 years. The remaining animal, also known to be at least 4 years old, had six lines, for an estimated age of 5 years. Up to 4 years of age, therefore, there appears to be a one-to-one relationship between the number of dark lines and age (Fig. 3).

Fig. 3. Number of adhesion lines present in known-aged and juvenile yellow-pine chipmunks. Age classifications are as in Fig. 1. Note that the animal with six dark lines was known to be at least 4 years old. Numbers beside each data point represent the number of animals included in that point.

All untagged males with total eye lens masses of <12 mg (10.0 ± 0.4 mg, $n = 3$) also had testes masses of <20 mg (15.0 ± 1.5 mg), whereas all tagged males had eye lens masses of >13 mg (16.3 ± 0.8 mg, $n = 6$) and testes masses of >40 mg (54.8 ± 3.8 mg), so the latter must have been adults. All untagged females with total eye lens masses of <12 mg (10.8 ± 0.1 mg, $n = 4$) showed no implantation sites, whereas all tagged females had eye lens masses of >13 mg (15.1 ± 0.3 mg, $n = 3$) and at least two visible dark spots, so the latter must have been adults. Nearly all of the untagged animals classified as juveniles by these criteria had only one dark line visible in their femoral sections. However, one animal that had been classified as a juvenile because of low eye lens mass (9.6 mg) and testes mass (13 mg) had two dark lines visible in the femoral sections, giving an estimated age of 1 year. We excluded this animal from the analysis, since it was untagged and we could therefore not be certain of its age.

Discussion

We have demonstrated that counting the thin, dark adhesion lines present in the femora of long-lived yellow-pine chipmunks is an excellent method to accurately determine age but that lens mass is not. In femoral sections, the number of dark lines, minus the first one found in all juveniles, indicates exactly the number of winters the individual has survived, up to at least 5 years. In contrast, lens mass (Fig. 1) was only able to reliably distinguish juveniles (approximately 3 months old) from adults (>1 year old), and jaw sections could not be used at all.

Morphological features such as body size or mass, eye lens mass, or reproductive traits may frequently be used to distinguish juveniles from adult individuals (Garshelis 1984; Malcolm 1992; Millesi et al. 1999; Kauhala and Soveri 2001). However, determining the age of adult animals is usually considerably more difficult. Adult animal age may be estimated by comparing certain characteristics of an individual with known mean values for certain age groups, but the variation inherent in these characteristics frequently precludes the accurate determination of the age of a single individual (Morris 1972). In the case of yellow-pine chipmunks,

juvenile animals typically reach adult body size by 3 months of age (Sutton 1992; Place and Kenagy 2000; Kenagy and Place 2000). In our study, juvenile and adult body masses did not differ, so this measure was not useful in determining or confirming age classifications.

Eye lens masses are commonly used as a crude estimator of a mammal's age, since they continue to grow after birth. Eye lenses grow fastest in an individual's first year of life; therefore, juveniles and adults can be readily distinguished. In the case of yellow-pine chipmunks, eye lens masses fell into two easily distinguished categories (juveniles and adults) that corresponded nearly perfectly to the counts of dark lines in the femora.

Sections of the mandible have also been used to age various mammalian species. However, we found that the mandible sections were not easily obtained in yellow-pine chipmunks. Whether this is due to the problem of lateral compression discussed by Klevezal and Kleinenberg (1969c) is unclear, but whatever the explanation, the difficulties encountered make this technique undesirable for use in this species.

In sciurids, previous studies suggest that the number of adhesion lines in bone represents the number of winters lived by the animal (Klebanova et al. 1966, cited in Klevezal and Kleinenberg 1969a). However, there may be inaccuracies in this method of age determination. Those animals born late in the fall would be hibernating with porous, unsolidified bone and thus missing the first expected adhesion line (Klevezal and Kleinenberg 1969a). Yellow-pine chipmunks breed only in the spring (Sutton 1992), so this potential missing line in certain individuals is not a concern in this species. However, it is necessary to consider the life history of the species being studied before relying on adhesion lines alone. Animals living for many years may also absorb old lines as new layers of bone are deposited (Klevezal and Kleinenberg 1969c). Chipmunks living in the wild have a maximum life expectancy of only 6–7 years, with most animals living only 2–3 years (Sutton 1992), so we did not expect old lines to be absorbed in a given individual's lifetime. In fact, one animal in our study had six lines visible, so adhesion lines are likely to be retained for at least 5 years.

Aside from difficulties with very long-lived animals, the use of bone annuli to age animals would be limited in its application to species that live through only one winter (e.g., voles and mice). Whereas it would distinguish individuals born before the most recent winter from those born after, it does not appear likely to prove useful in resolving ages in any finer detail. However, since bones are slowly degraded after death relative to soft tissues, and are not readily digested by predators, the method proposed in this paper would be particularly useful for determining the age of specimens for which soft tissue is in poor condition. For example, long bones remaining at the site of kills by predators, found in the pellets of birds of prey, from animals killed by vehicles, or from museum specimens could be used to determine the age of an individual at death to within 1 year.

Although a single animal out of 16 animals was discovered in this study whose classification from eye lens and testes mass did not agree with that from adhesion lines, given the reliability in determining the age of adult animals, we are confident that such a small degree of potential error is

acceptable. It is possible that this animal was a juvenile who had experienced inordinate stress at some point in his postweanling life that slowed growth and produced the extra adhesion line (Klevezal and Kleinenberg 1969b). On the other hand, it is possible that it was in fact 1 year old but had experienced underdevelopment of the soft tissues. Tagging juveniles and recapturing some later in their year of birth and others the following year would determine with a greater degree of certainty the accuracy of the method described in this paper. However, given the accuracy of the age estimates for older animals, it seems most likely that this technique does in fact reliably indicate the age of an individual.

Ideally, a small population of known-age individuals should be examined to calibrate any technique before it is used to age a given species for the first time. This study demonstrates that once calibration is done, femoral sections are more accurate than eye lens masses and mandibular sections in yellow-pine chipmunks. The use of femur adhesion lines for age determination is likely to be applicable to other seasonally breeding rodents with life expectancies of several years.

Acknowledgements

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