Determining kangaroo age from lens protein content

Robert C. AugusteynA, Graeme CoulsonB and Kerry A. LandmanC

A The Vision Cooperative Research Centre, University of New South Wales, Randwick, NSW 2052, Australia.
B Department of Zoology, University of Melbourne, Vic. 3010, Australia.
C Department of Mathematics and Statistics, University of Melbourne, Vic. 3010, Australia.

Abstract

Kangaroos can be aged by external morphometrics only when young (pre-weaning). Indices of molar progression are used to estimate the age of older kangaroos, but that method rests on a number of tenuous assumptions. This study explored the use of the eye lens as an alternative method for determining age. Body mass, foot size, arm length and wet mass of the lens were measured in 40 red (Macropus rufus), 476 western grey (M. fuliginosus) and 57 eastern grey (M. giganteus) kangaroos, ranging in age from 3 days to ~20 years. Total protein contents were determined for 556 lenses from these animals. Body mass and limb dimensions increase with age, at different rates for males and females, but the wet mass and protein content of lenses are independent of sex. Furthermore, the lens data approach their asymptote later than any of the other measurements, making them more reliable for aging older animals. The relationship between total protein contents (in milligrams) and age (in years estimated from molar index) can be described with a single equation for all three species over the whole age range from newborn to adult.

Introduction

Kangaroos are harvested commercially in the rangelands of the four largest Australian States (Shepherd and Caughley 1987) and, at a smaller scale, overabundant populations have been managed by culling and fertility control in a number of nature reserves (Coulson 2002). Adequate predictive models of these populations require data on the age structure and age-specific performance of the population (Pople and Cairns 1995). There is thus a need for an efficient and accurate method for aging kangaroos.

Young kangaroos can be aged readily by linear body measurements. In known-age eastern grey kangaroos (Macropus giganteus), for example, Poole et al. (1982a) showed that head and foot length predict age from birth to the end of pouch life (approximately 11 months) quite accurately, and could be used with less confidence until the animal reached 2 years of age. Body size, particularly mass, becomes quite variable as kangaroos grow. The standard method for aging kangaroos above 2 years of age is by measurement of molar progression: molars erupt at the posterior end of the molar row and progress towards the diastema, coming into occlusion then being shed as they wear. Kirkpatrick (1964, 1965) defined the molar index (MI) as the number of molars (including fractions) in the maxillary row that have moved past an arbitrary reference line drawn between the anterior rims of the orbits, and showed that log(true age) was linearly related to MI, with a species-specific slope. This method has been used in a number of studies of age structure in kangaroos (e.g. Norbury et al. 1998; Coulson 1989).

Measurement of MI creates a number of practical difficulties. A level of skill is needed to identify molars in their eruption sequence, distinguishing them from molariform deciduous premolars and occasional supernumerary molars (Kirkpatrick 1965). Accurate measurement requires a palatal view of the orbits and the molar rows in the maxillae, which
can be obtained only by radiography in living animals (Kirkpatrick 1965). Although costly, radiographs can also be taken of dead specimens. Alternatively, heads can be dissected to remove the mandibles and reveal the orbital rims, which is a time-consuming process. Heads can also be cleaned by boiling, or by natural decomposition in soil, but this takes longer than dissection.

The use of MI also raises several methodological issues. Sanson (1982) has shown that molar progression is a function of chewing forces, not age per se. The relationship between MI and age reported by Kirkpatrick (1965) was derived from captive animals. Food intake in captivity has been shown to be less than in free-ranging conspecifics of many herbivores (e.g. Nugent et al. 2000), so the apparent age of kangaroos in the field is likely to be higher than the true age, but has never been calibrated. Kirkpatrick’s (1965) relationship between MI and age also assumes that the rate of progression is equivalent for males and females, but dietary segregation between the sexes could result in different chewing rates and thus influence apparent age. Newsome et al. (1977) reported this effect in free-ranging agile wallabies (M. agilis), but it has not been examined in kangaroos. In addition, Kirkpatrick (1965) determined MI versus age for only two kangaroo species. In the absence of a specific aging scale for the western grey kangaroo (M. fuliginosus), researchers (e.g. Robertson 1986; Norbury et al. 1998) have been forced to assume that the rate of progression is equivalent in eastern and western grey kangaroos, which are sibling species and similar in most respects (Kirsch and Poole 1972), but which may not have identical patterns of molar progression.

A better approach may be to use lens properties. It was first noted by Smith (1883), and subsequently confirmed in numerous studies (e.g. Dudzinski and Mykytowycz 1961; Friend 1967a, 1967b; Hockwin et al. 1971; Pierscionek and Augusteyn 1992) that lens growth is continuous throughout life. Since it does not discard old cells or their contents, the lens contains a record of its own growth. Moreover, lens growth is largely unaffected by fluctuations in the external environment and nutrition (Kaufman and Norton 1966; Friend 1967c). These features are of great value for age determination and have led to a number of attempts to determine age from the wet mass of the lens (Fink et al. 1970; Bours et al. 1983; Pierscionek and Augusteyn 1992). While wet mass of lenses, freshly removed from the eye, can be effectively used for estimating ages in laboratory and domestic animals (Fink et al. 1970; Hockwin et al. 1971, 1979; Pierscionek and Augusteyn 1992), the variability within samples collected in the field is generally too great to permit their use for accurate age determination. This can be attributed largely to post-mortem water movement, especially during freezing and thawing of eyes, or to prolonged storage. Measurements that are largely independent of lens water content would be preferable. Such an approach was introduced by Lord (1959), who showed that age could be determined from the mass of the lens after fixation and oven-drying. The method has been successfully adopted for determining the ages of several species, including wild rabbits in Australia (Dudzinski and Mykytowycz 1962), but it takes ~2 weeks to obtain data and the drying process may not always be consistent, leading to errors. Eliminating the need to fix and dry lenses would simplify and speed up the determination of age.

Since most of the dry mass of the lens is protein, direct estimation of protein content may provide a better method for determining age. Previous investigators have explored this possibility by measuring soluble and/or insoluble protein contents (Dische et al. 1956; Birney et al. 1975; Ludwig and Dapson 1977; Pierscionek and Augusteyn 1992). Although insoluble protein content does increase with age, the difficulties in effectively separating insoluble and soluble proteins as well as alterations in the solubility of proteins during
Determining kangaroo age from lens protein

Aust. J. Zoology

handling and storage can significantly alter the amount (Coghlan and Augusteyn 1977). Total protein content of the lens would provide a more reliable measure of growth. Since there is no discernable protein turnover in the mature fibre cells, which account for the bulk of the tissue, and since each fibre cell will contain the same amount of protein at maturity, lens protein content will be directly related to the number of fibre cells laid down in the lens. The present study explores the correlation of lens protein content with ages estimated from the Molar Index.

Study Area and Methods

Eyes were collected from western grey kangaroos and red kangaroos (M rufus) culled at Hattah-Kulkyne National Park, Victoria, in 1992 (n = 209) and 1999 (n = 245) and from pouch young (n = 64), obtained in the 1999 cull. Culling was conducted by spotlight at night by professional shooters under the supervision of Parks Victoria staff. Kangaroos were shot in the head, brought to a disposal pit and given a numbered ear tag on arrival. Data collected from sub-adults and adults were species, sex, age class, reproductive status, body mass, and length of arm (males only), leg and foot. Pouch-young were removed, and their sex, head length and foot length recorded. Eyes were removed by dissection, stored at ambient temperature (10–15°C) up to a maximum of 12 h and then at 4°C if they could be processed within 24 h. Those that could not be processed in this time were immediately frozen and thawed no more than 5 days later for removal of the lens. After removal of the eyes, heads were severed at the neck and placed in individual nylon mesh bags. The bags were buried in sandy soil, extracted several months later and cleaned for measurement of MI.

Lenses from 44 eastern grey kangaroos were obtained from a cull conducted by consultant biologists at Government House, Canberra, in 1993. Animals were captured using tranquilliser darts then euthanased by lethal injection. Lenses from 13 eastern grey kangaroos culled at Portland Aluminium Smelter, Victoria, in 2001, were also included in the study. Culling was conducted by smelter staff. Protocols for data collection at both sites were the same as at Hattah-Kulkyne.

Ages of pouch-young were estimated from head and foot length, using sex-specific growth equations calculated by Poole et al. (1982a) for western grey kangaroos, and read off graphs presented by Sharman et al. (1964) for red kangaroos. Ages of older animals were calculated from MI, using Kirkpatrick's (1965) equation for eastern grey kangaroo for both grey kangaroo species, and Kirkpatrick's (1970) equation for red kangaroos.

Lenses were removed through an incision made in the limbal region of the eye, freed from adhering vitreous and pigmented tissues, gently blotted dry and weighed. Obviously damaged lenses were discarded while most of the intact lenses were placed in 5.0 mL phosphate-buffered saline (PBS) and homogenised by sonication. Since kangaroo lens proteins tend to precipitate within a short time after extraction, up to 2.0 mL of 6M urea in PBS were added to each extract to maintain solubility. This did not affect the protein assay. Protein contents of the extracts were then determined by the Lowry method as described previously (Pierscionek and Augusteyn 1992). Lenses that appeared to be small for their age, and in which the mass fraction of protein was greater than 0.45, were judged to be damaged and the data discarded. Usable data were obtained for 751 lenses.

The protein versus age data were fitted to a variation on a logistic-type equation using a non-linear least-squares algorithm (Leven-Marquardt algorithm: Press et al. 1987) for the unknown parameters.

Results

Data were obtained from 531 red, western grey and eastern grey kangaroos, ranging in age from 3 days to ~20 years. Males and females were almost equally represented. Body mass, leg length, foot size and lens mass for the red and western grey kangaroos are presented in Fig. 1a–e, as a function of age determined from head length for the pouch young and molar index for the others. Fig. 1f–j expands the portions of the plots that contain data from animals aged over 4 years, together with the line of best fit.

All body parameters were found to increase with age. Some variability was observed, as might be expected from individual differences. No significant differences were detected between red and western grey kangaroos. Males and females grow at different rates, with
males attaining much greater body sizes at the same age than females. As can be seen in the
data for body mass, leg length and foot length (Fig. 1a–c), this becomes very apparent by
2 years. By ~5 years, these parameters appear to have reached their maxima and, as can be
seen from Fig. 1f–h, remain constant or decrease thereafter.

No such divergence is observed in the masses of the 725 red and western grey kangaroo
lenses examined (Fig. 1d). Males and females appear to have the same-sized lens at the
same age, regardless of body size. Although there is some variability, especially with lenses
from older animals and with lenses that had been stored, it is clear from Fig. 1d and Fig. 1i
that lens size increases asymptotically with age.

Total protein contents were determined for the lenses and the data are presented in
Fig. 1e. They appear less variable than any of the other parameters measured. The protein
content of the lens also increases asymptotically with age, suggesting that there may be a
finite limit to the amount of protein that can be accommodated in the adult lens. Extrapolations to infinite time from double reciprocal plots of the data suggested that this
limit might be ~450 mg. Again, no differences could be detected between males and
females or between the red and western grey kangaroos. For the eastern grey, the
extrapolation suggested a limit of ~465 mg.

The effect of storage was examined by comparing the protein contents of 21 western
grey kangaroo lenses that had been stored for ~1 year at –20°C with those from freshly
assayed lenses. No differences were observed between the stored and fresh lenses for
animals with the same MI. However, examination of lenses removed from 44 eastern grey
kangaroo heads that had been stored for 8 years and repeatedly thawed and frozen indicated
that as much as 30% of the proteins had been lost. These lenses were not included in the
present analyses.

Curve fitting of lens protein data from 177 western grey kangaroos collected in the 1999
cull (256 lenses) was undertaken to determine the relationship between lens protein content
and age. Since there were no gender differences, all data were combined. Log(TPC) is
plotted against log(age) (from MI or head length) in Fig. 2.

Two phases are evident in the growth (accumulation of protein) curve. The first, rapid
phase corresponds to growth in the pouch while the second, much slower phase represents
growth after the animal leaves the pouch. The transition from rapid to slow growth occurs
over a relatively short period, starting after 7 months of age and finishing before 1 year.

An excellent fit of the data (correlation coefficient $R = 0.9963$) over the whole age range
was obtained with the relationship

$$\text{Log}_{10}[\text{TPC}] = a - \frac{b}{1 + cA}$$

where TPC = total protein content of a lens (in milligrams), A = age in years, $a = 2.66$, $b =
4.66$, and $c = 12.57$. The curve corresponding to this fit is also shown in Fig. 2. The 95%
confidence interval of the parameters $a$, $b$ and $c$ are, respectively, $2.66 \pm 0.023$, $4.66 \pm 0.10$
and $12.57 \pm 0.820$.

Fig. 1. Changes with age (determined from the molar index or head length), in (a) kangaroo body mass,
(b) foot length, (c) leg length, (d) lens mass and (e) lens protein content, are presented for red (circles) and
western grey (triangles) kangaroos. Female data are shown with closed symbols and male data with open
symbols. The portions of the plots containing data for animals older than 4 years are expanded in the
corresponding figures (f–j). The line of best fit (linear for (f–h); log for (i–j)) is included for
illustration purposes only.
The red kangaroo data could also be adequately described with this equation and the same values for the constants. For the 26 eastern grey kangaroo lenses collected in 1999 and 2001, constants of 2.66, 4.66 and 10.00 were obtained ($R = 0.9983$).

**Discussion**

Various physical measurements have been used to estimate age in kangaroo populations. These include body mass, the length of head, foot, leg and arm, and the molar index. Most of these suffer from the disadvantage of requiring different relationships for different ages and sexes. Most are also affected by environmental factors and are unreliable for ages greater than 2 years.

Our data indicate that protein content of the kangaroo lens could provide a much more reliable estimate as long as the samples have been properly stored. Total protein content increases asymptotically as a function of age and the relationship can be accurately described with a single logistic-type equation (Equation 1) for all ages, including prenatal, and both sexes.

This type of equation was also used by Dudzinski and Mykytowycz (1961) and Myers and Gilbert (1968) when fitting the dry mass of the lens with age for wild rabbits in Australia.

Rearrangement of Equation (1) generates Equation (2), which can be used for estimation of age from the protein content.

$$A = \frac{1}{c} \left( \frac{b}{a - \log_{10}[TPC]} - 1 \right)$$

Since we are fitting with a logistic-type curve, it may be hypothesised that the dynamics of the protein growth follow a simple differential equation. From equation (1) the total
Determining kangaroo age from lens protein content at zero age is \( \log_{10}[TPC(0)] = a - b \). For ease, \( Y(A) \) can be defined to be a measure of TPC at age \( A \) relative to TPC at age zero:

\[
Y(A) = \log_{10}[TPC(A)] - \log_{10}[TPC(0)] = \log_{10}\left(\frac{TPC(A)}{TPC(0)}\right)
\]  

(3)

It is easily verified that this function satisfies the differential equation

\[
\frac{dY}{dA} = \frac{Y}{A} \left(1 - \frac{Y}{b}\right)
\]  

(4)

Writing \( X = \log_{10}[A] \), Equation (4) becomes

\[
\frac{dY}{dX} = rY(1 - \frac{Y}{b}).
\]  

(5)

where \( r = \log_{e}[10] \). This equation is just the standard logistic equation (Murray 1990).

Equations (4) and (5) may be interpreted as saying that the growth of protein content in a lens is limited by space or resources available and this gives rise to a carrying capacity. Furthermore, the growth rate decreases with age. Why the protein growth for kangaroo and wild rabbit lenses should satisfy such a simple generic equation is unknown at this stage.

As noted, the fitting curve has an excellent correlation with the data. Several additional pieces of information can be deduced from this fit. As age increases, the fit suggests an asymptotic value \( \log_{10}[TPC(\infty)] = a \), yielding \( TPC(\infty) = 452 \text{ mg} \) for the red and western grey kangaroos and 465 mg for the eastern greys. These match well with the extrapolations above (450 and 465 mg). The difference in the asymptotic values indicates that the eastern grey kangaroo lens is larger than that of the other species.

The curve, as age decreases towards zero, yields another asymptotic limit of \( \log_{10}[TPC(0)] = a - b = -2.00 \), hence \( TPC(0) = 0.01 \text{ mg} \). This is the limiting value of the protein lens content at the birth of pouch young. Of course, lens growth commences prior to birth. An estimate of the time this occurs may be obtained from extrapolation of the protein content to zero. As TPC approaches zero, \( A \) approaches \(-1/c\). The limiting value for the western grey kangaroo is \(-29 \pm 2 \text{ days} \) relative to birth. This value seems quite reasonable, given that the gestation time for this species is 30.6 days. For the limited number of specimens of eastern grey kangaroos, \( 1/c = 36 \), close to the 36.4-day gestation period. The agreement between the two sets of numbers suggests that the time of fertilisation should be considered as the time that lens growth commences, rather than the time that lens proteins are first produced. Lens-specific protein synthesis can first be detected in the pit cells of the developing rat lens vesicle at about Day 12, one day before the separation of lens and skin ectoderm (McAvoy 1981). Separation of the lens from skin ectoderm occurs at about Day 24 in marsupials (Dunlop et al.1988), suggesting that crystallin synthesis may commence at about Day 22, corresponding to 17 days before birth.

Thefitting curves of Dudzinski and Mykytowycz (1961) and Myers and Gilbert (1968) yielded corresponding values of \(-41\) and \(-57 \text{ days} \) respectively, for the wild rabbit, which has a gestational period of 30–35 days. These values also suggest fertilisation as the commencement of lens growth although some re-evaluation of the rabbit fitting curves seems warranted. The similarity between the fitting curves for wild rabbit and kangaroo suggests that the same relationship may also apply for other species. This possibility is currently under investigation.
As pointed out earlier, the use of MI for determining age has a number of difficulties. In practice, this can result in long delays before data are available and occasional over-estimates of age, mainly with older animals. Close examination of the data described in Fig. 1 suggests that some of the ages determined from MI are not consistent with the external morphometrics. For example, the points at apparent molar ages of 17.5 and 18.7 years should probably lie around 6–7 years. These individuals may have had abnormal dentition, as described by Kirkpatrick (1965), or their rate of molar progression may have uncoupled from tooth wear, as reported by McArthur and Sanson (1988).

By contrast, lens protein content appears to present no major difficulties. In all species examined so far, protein content or dry weight continue to increase throughout life (e.g. Dische et al. 1956; Dudzinski and Mykytowycz 1961; Friend 1967a, 1967b; Ludwig and Dapson 1977; Pierscionek and Augusteyn 1992) and are unaffected by nutritional status or sex (Kaufman and Norton 1966; Friend 1967c; Birney et al. 1975; Bours et al. 1983). It can also be measured rapidly and accurately. It is concluded that, where there is access to appropriate laboratory facilities, measuring lens protein contents could be the preferred method for determining kangaroo age. However, it will first be necessary to test the conclusions from this study with data from known-age animals.

Acknowledgments
The authors are grateful to David Major, Phil Murdoch and their staff at Hattah-Kulkyne National Park, Pat Garrett, David Middleton and Bryan Walters at Government House, and John Hill and Ron Jeffries at Portland Aluminium. We also thank Ian Beveridge, Emily Bolitho, Emily Clarke, Abigail MacFarlane, Jane McKenzie, Leanna Rosier, Clorinda Schofield and Sally Troy for assistance with the measurements and collection of tissues. Dr Yvonne Stokes, University of Adelaide, made valuable input into the curve fitting. This work was part of the research activities of the Cooperative Research Centre for Eye Research and Technology at the University of New South Wales.

References


Determining kangaroo age from lens protein

Aust. J. Zoology 493


Manuscript received 13 February 2002; accepted 2 September 2003