

Telomere Length Shortens with Body Length in *Alligator mississippiensis*

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Abstract - In *Alligator mississippiensis* (American Alligator), body length increases with age, but body length can be used as an accurate estimator of age only up to about 6–7 years, when growth rates slow considerably. Telomeres are repetitive DNA sequences that cap the ends of each chromosome. Telomeres shorten with age in most animals, but telomere shortening has not been examined in reptiles. We measured telomere length in erythrocytes of *A. mississippiensis* varying between ≈ 5 and 240 cm in body length and found a negative relationship between telomere length and body length ($P < 0.01$). Assuming that erythrocyte telomeres continue to shorten with time, even after growth rate declines, those individuals with the shortest telomeres should be the oldest members of the population. This method of estimating age, even in animals of similar body size, should allow questions about age structure and senescence to be addressed.

Introduction

Knowledge of population dynamics may be a critical component in the conservation and management of species. Age structure can be a major determinant of population growth potential, but age is often difficult to measure. Likewise, attempts to measure senescence as either age-specific decreases in survival or fecundity depend on knowing the age of individuals. For *Alligator mississippiensis* Daudin (American Alligators), current methods of estimating age include tagging young animals or using age-specific character measurements, such as body length and skeletochronology (Tucker 1997). Each of these methods has shortcomings. For example, web tags can be lost or tagged animals may leave the study area, and the validation of skeletochronology in reptiles is labor intensive (Snover and Hohn 2004, Tucker 1997, Wayne and Gregory 1998). Measurement of body length to estimate age can be accurate in young animals, but becomes less accurate when growth rates change with age, vary with local conditions, or differ between sexes as they do in American Alligators (Chabreck and Joanen 1979, Rootes et al. 1991).

Telomere length has been proposed as an estimator of age in birds (Haussmann and Vleck 2002) and humans (Tsuji et al. 2002), and it would be useful to know whether telomere lengths could also be used to estimate age in alligators. Telomeres are short repeated sequences of DNA found at

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the ends of linear eukaryotic chromosomes that, along with associated nucleoproteins, function in stabilizing chromosomal end integrity (Blackburn 2001). Telomeres in somatic tissues tend to shorten with age, because DNA polymerase is unable to replicate completely the 3' end of linear DNA with each cell division (Watson 1972) and because of the effects of oxidative stress (von Zglinicki 2000). As it is not known whether telomeres shorten over time in long-lived reptiles such as alligators, our objective was to determine whether telomeres are shorter in larger (and thus presumably older) animals than in smaller (presumably younger) animals.

Methods

Animals and blood collection

We collected blood from 26 free-ranging alligators at the Rockefeller State Wildlife Refuge in Grand Chenier, LA and from eight embryos from three clutches collected in the wild and incubated at the refuge headquarters. None of the free-ranging animals had been reared in captivity and released as part of Louisiana's egg-ranching program, a process that accelerates early growth (Elsey et al. 2000). Six of the adult females were caught at their nest, so we also know their clutch sizes. We knew the incubation age of the embryos (23 to 45 days), but did not know ages of the wild-caught individuals. Total body lengths (TBL) were measured from the snout tip to tip of tail. Blood was drawn from the spinal vein in wild-caught alligators (Zippel et al. 2003) or from the umbilical vein in embryos. After marking, captured animals were released. Approximate blood volumes collected were: 100 μ l from embryos, 1 ml from animals < 50 cm, and 10 ml from larger animals. Blood samples were immediately placed in ice-cold 2% EDTA buffer, then later packed in ice and shipped to Iowa State University. Samples were kept at 4 °C until the time of DNA extraction, which occurred within three weeks of collection.

DNA isolation, digestion, and electrophoresis

We extracted DNA from erythrocytes (about 4 μ l) in 100-ml 0.8%-agarose plugs using a CHEF DNA plug kit (Bio-Rad, Hercules, CA) to avoid DNA shearing. Plugs were incubated overnight at 50 °C in 10 ml proteinase K, followed by a three-hour incubation at 37 °C with PefaBloc to deactivate residual proteinase K. We digested the DNA using a mixture of three restriction enzymes: *Hinf I*, *Hae III*, and *Hind III* (50 U each) for 7 hours at 37 °C. Digested plugs were loaded onto a 0.8%-agarose gel and resolved using pulsed-field gel electrophoresis (CHEF-DR II; Bio-Rad) for 21 hours at 14 °C (3 V/cm with a switching time of 0.5–7.0 s and a current of 63–66 mA). After electrophoresis, the gel was hybridized to a ³²P-labeled oligonucleotide (TTAGGG)_n that binds to the single stranded portion of each telomere restriction fragment (TRF). Radioactive signaling of the ³²P-labelled TRFs was detected by a phosphor imager system (Typhoon 8600, Molecular Dynamics, Sunnyvale, CA). Densitometry (ImageQuant V 1.2 and Telometric) was used to estimate mean TRF length of each telomeric

smear (Fig 1). On each gel, we ran two different molecular markers, ranging from 1 to ≈ 50 Kb (monocut and 1 Kb +). To estimate the mean length of the TRFs in each lane (also referred to as telomere length), we used the formula: $TRF = \sum(OD_i L_i) / \sum(OD_i)$, where OD_i is the densitometry output at position i , and L_i is the length of the DNA (bp) at position i . A single blood sample was randomly selected to run in three lanes of each gel to test the uniformity of DNA migration through each gel and to calculate inter-and intra-gel variability. Intra- and inter-gel coefficients of variation were low (1.0% and 3.6% respectively), so the data from the two gels were combined.

We used linear regression to determine whether telomere length varied with TBL, and analysis of covariance to examine the effect of sex on telomere length, using TBL as the covariate.

Results

We analyzed blood samples from 34 alligators ranging in size from ≈ 5 to 240 cm, including eight embryos and 26 wild-caught alligators, of which 12 could be identified as males and 12 as females. Mean telomere lengths ranged from 34 to 27 Kb in animals that varied from ≈ 5 (embryos) to 240 cm in body length. There was a significant decrease in mean telomere length with increasing total body length: $TRF = 30.9 - 0.008TBL$; $F_{1, 32} = 6.9$; $P < 0.01$, $r^2 = 0.18$ (Fig. 2). Animals lost on average 8 base pairs of

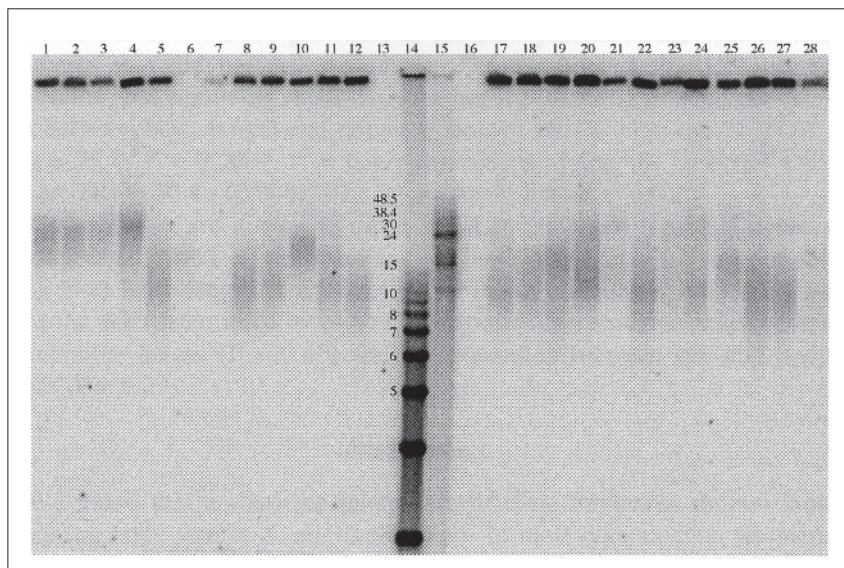


Figure 1. Southern blot showing telomere restriction fragments from erythrocytes of *Alligator mississippiensis* of different body lengths. Molecular-size markers are shown in lanes 14 and 15 with the size of the markers (in Kb) indicated just to the left. Hybridization of the telomeric probe to the single-stranded overhang of the TRFs produces a smear because telomeres from different chromosomes and different cell populations vary in length.

telomere per cm of body growth. For the embryos, three individuals had telomeres that did not fall within the 95% confidence interval for the regression, but removing these data did not significantly change the relation between body length and telomere length: $TRF = 31.4 - 0.011TBL$; $F_{1,29} = 25.7$; $P < 0.0001$, $r^2 = 0.47$; nor did removing the embryos altogether: $TRF = 30.8 - 0.008TBL$; $F_{1,24} = 8.0$; $P < 0.01$, $r^2 = 0.25$.

Growth rates are known to differ between male and female alligators (Rootes et al. 1991), so we examined telomere length as a function of sex using total body length as a covariate for those individuals for whom we knew the sex (determined by cloacal palpation). There was no effect of sex on telomere length ($P = 0.80$), and no interaction between sex and body length ($P = 0.23$).

Discussion

We found that erythrocyte telomere restriction fragments shorten with increasing body size in *A. mississippiensis*. In most taxa studied, telomere restriction fragments are shorter in older animals (Allsopp et al. 1992, Delany et al. 2000, Hastie et al. 1990, Haussmann et al. 2003). Peripheral blood cells do not divide, so these telomere lengths presumably reflect the telomere lengths of the hematopoietic stem cells. Hematopoietic stem cells may express some

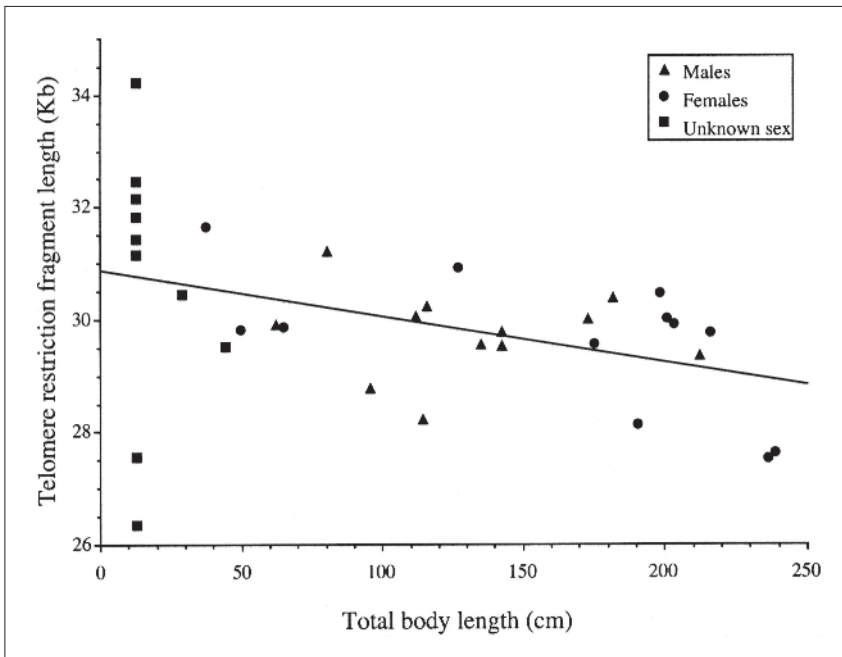


Figure 2. Relationship between total body length and telomere restriction fragment length measured in erythrocytes obtained from *A. mississippiensis*. The least squares equation for the fit line is $TRF = 30.9 - 0.008TBL$; $F_{1,32} = 6.9$; $P < 0.01$, $r^2 = 0.18$.

telomerase, an enzyme responsible for maintaining telomere length, but they still lose telomeric repeats over time (Vaziri et al. 1994). Blood cells typically have a high turnover rate (Chang and Harley 1995, Cline and Waldmann 1962), and thus, blood is an excellent candidate tissue for aging studies.

We did not know the age of these free-living animals, but alligator growth rates have been previously studied in coastal Louisiana wetlands (Chabreck and Joanen 1979, Elsey et al. 1992, Rootes et al. 1991). Growth rates of males and females do not differ significantly (≈ 36 cm/year) until about 3 years of age, when animals have reached about 1 m in length (Elsey et al. 1992). Growth rate then appears to decline more rapidly in females than in males (Rootes et al. 1991). Thus males in the population can reach a larger maximum size than females (> 4 m for males, ≈ 2.5 m for females), and for adult animals of the same size, females are probably older than males (Rootes et al. 1991). Given the different ages of males and females of the same size, we predicted there should be an effect of sex on the relationship between telomere length and body length. We did not find an effect of sex in our data, probably because we did not have enough very large animals (particularly males) to have the needed power to test this prediction.

Senescence is usually characterized by age-related decreases in survival and fecundity. Others have suggested that long-lived turtles do not undergo senescence (Congdon et al. 2003, Girondot and Garcia 1999). Senescence in alligators, which can live for over 50 years (R.M. Elsey, unpubl. data) is poorly studied, because it is difficult to determine age in the oldest individuals in the population. Clutch sizes for *A. mississippiensis* at the Rockefeller Wildlife Refuge can vary from 2 to 58 eggs, averaging about 39 (Joanen 1969). Small females that have just reached sexual maturity produce small clutches (Giles and Childs 1949), and there is a positive relationship between clutch size and body size in general for this population (R.M. Elsey, unpubl. data). In our samples, the two largest females with the shortest telomeres had the largest clutch sizes (40 and 41 eggs), and the shortest adult female with the longest telomere had the smallest clutch size (26 eggs). Some mature females have been described as barren (Joanen and McNease 1980), but it has been difficult to determine whether clutch size decreases in the oldest animals (see Joanen 1969). Growth is very slow after about 20 years of age in females, making it difficult to identify the oldest individuals in the population using only size as a reference. Turnover rate of red blood cells in *A. mississippiensis* can be as short as ≈ 180 days at warm temperatures and decreases at cold temperatures along with growth and metabolism (Cline and Waldmann 1962). Assuming that the telomere-shortening rate of erythrocytes we demonstrated can be extrapolated across the life span of individuals as their blood cells continue to be replaced, the oldest animals should have the shortest telomeres. This technique will not be useful to provide a particularly accurate estimate of age of an individual; however, it should make it possible to distinguish older individuals from younger individuals, even when they are approximately the same body length. If blood

samples can be collected from large females with known clutch sizes, assigning relative age based on telomere length should allow us to identify age-related decreases in fecundity, if they exist.

The telomere lengths of the eight embryos we measured were quite variable, although generally longer than in the older alligators. In fact, the range in telomere length of the embryos was greater than in the other 26 individuals. These embryos were all alive when sacrificed between 23 and 45 days of incubation, but it is uncertain whether they all would have hatched. The eggs originated from three clutches, but there was no effect of clutch of origin or days of incubation on telomere length ($P > 0.38$). Embryos of many organisms exhibit high activity of telomerase, an enzyme that can maintain telomere length in the face of very high cell division (Xu and Yang 2000), and telomere length may actually increase during development (Schaezlein et al. 2004). Telomere dynamics during embryonic development may differ from that in post-hatching individuals because of a different balance between cell replication rate and telomerase activity (Forsyth et al. 2002), but this needs further study.

Telomere restriction fragment lengths in *A. mississippiensis* are long relative to those in many birds and mammals (Frenck et al. 1998, Haussmann et al. 2003, Hemann and Greider 2000), although the domesticated lab mouse *Mus spretus* Lataste is known to have ultralong telomeres (Kipling and Cooke 1990) as are some birds (Delany et al. 2000). Girandot and Garcia (1998) reported telomere lengths in *Emys obicularis* Lineaus (European Freshwater Turtles) of about 20 Kb, while Paitz et al. (2004) reported telomeres in *Chrysemys picta* Gray (Painted Turtles) of about 60 Kb. Recently, telomeres of only about 14.5 Kb were reported in *Thamnophis elegans* Smith and Smith (Garter Snakes) (Reid et al. 2004). In general, chelonians and crocodylians live considerably longer than squamates, so there may be a functional relationship between absolute telomere length and lifespan, although this has not been found within birds and mammals (Kipling and Cooke 1990, Vleck et al. 2003). The measured length of telomere restriction fragments, however, varies with technique used (Delany et al. 2000, Haussmann et al. 2005), so caution needs to be used in making interspecific and cross-study comparisons.

In conclusion, we have demonstrated that erythrocyte telomeres shorten with increasing body length in *A. mississippiensis*. It seems reasonable that the oldest individuals in the population will have the shortest telomeres, and identification of these individuals should allow us to address questions regarding whether potentially long-lived crocodylians exhibit senescence in the wild.

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