



Ambiguities in using telomere length for age determination in two North American bat species

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The age of an animal, determined by time (chronological age) as well as genetic and environmental factors (biological age), influences the likelihood of mortality and reproduction and thus the animal's contribution to population growth. For many long-lived species, such as bats, a lack of external and morphological indicators has made determining age a challenge, leading researchers to examine genetic markers of age for application to demographic studies. One widely studied biomarker of age is telomere length, which has been related both to chronological and biological age across taxa, but only recently has begun to be studied in bats. We assessed telomere length from the DNA of known-age and minimum known-age individuals of two bat species using a quantitative PCR assay. We determined that telomere length was quadratically related to chronological age in big brown bats (*Eptesicus fuscus*), although it had little predictive power for accurate age determination of unknown-age individuals. The relationship was different in little brown bats (*Myotis lucifugus*), where telomere length instead was correlated with biological age, apparently due to infection and wing damage associated with white-nose syndrome. Furthermore, we showed that wing biopsies currently are a better tissue source for studying telomere length in bats than guano and buccal swabs; the results from the latter group were more variable and potentially influenced by storage time. Refinement of collection and assessment methods for different non-lethally collected tissues will be important for longitudinal sampling to better understand telomere dynamics in these long-lived species. Although further work is needed to develop a biomarker capable of determining chronological age in bats, our results suggest that biological age, as reflected in telomere length, may be influenced by extrinsic stressors such as disease.

Key words: Ageing, *Pseudogymnoascus destructans*, qPCR, relative telomere length, telomere lengthening, vespertilionidae, wild

Determining the age of individuals is critical to understanding mammalian demography, population ecology, and conservation or management needs. Chronological age, or time elapsed since birth, can influence the likelihood of reproduction or mortality, which in turn influences population growth rate (Stearns 1992; Brunet-Rossinni and Austad 2004; Roach and Carey 2014). Ecological factors interact with chronological age to determine physiological state, or biological age, which can lead to differences in development, reproduction, and longevity among individuals in a population (Stearns 1992; Dunshea et al. 2011; Jarman et al. 2015). Both chronological and biological age are needed to fully understand the ecology (Stearns 1992) and conservation (Roach and Carey 2014) of a species. Bats are a

particularly interesting taxon for studying age because they are long-lived for their body size, generally have low extrinsic mortality, and in some lineages have evolved behavioral or genetic mechanisms to resist viruses and suppress cancer (Wilkinson and Adams 2019). Bats are, however, susceptible to some diseases. The arrival and spread of white-nose syndrome, a fungal disease, has led to the collapse of bat populations throughout eastern North America (Frick et al. 2010), bringing new urgency to the need to better understand the demographics of remnant populations.

Bats pose a unique challenge to demographic studies because while they are long-lived, they do not have reliable external indicators of age (Brunet-Rossinni and Wilkinson 2009).

Two well-studied vespertilionid species in North America, the little brown bat (*Myotis lucifugus*) and the big brown bat (*Eptesicus fuscus*) have recorded lifespans of 34 years (Davis and Hitchcock 1995) and 19 years (Hitchcock 1965), respectively. These species may regularly live at least 10 years without signs of senescence in reproductive activity (Hall et al. 1957; Paradiso and Greenhall 1967) and there are multiple records of little brown bats living over 20 years (Keen and Hitchcock 1980; White et al. 2019). Although these species can be identified as juveniles during their first summer (Kunz and Anthony 1982), there is no reliable way to determine age after their first year. For example, tooth wear is not a dependable predictor of age in little brown bats (Hall et al. 1957) and has never been successfully calibrated against samples of known-age individuals for *Eptesicus* spp. (Christian 1956; Hood et al. 2002; Gol'din et al. 2018). Incremental lines of cementum and dentin in teeth or bones likewise have not been well calibrated (Phillips et al. 1982) and also are impractical for studies of live animals. Without external indicators of age, long-term banding or passive integrated transponder (PIT) tagging efforts currently are the only options for determining the chronological age of individuals and studying age-related changes in survival and reproduction.

While external markers have been unsuccessful for determining age in little and big brown bats, genetic markers such as telomere length offer a potential solution. Telomeres have been widely studied as genetic markers of both chronological (Jarman et al. 2015) and biological age in a variety of taxa (Monaghan 2010), but only recently in bats (Foley et al. 2018). Telomeres are made up of the tandemly repeated nucleotide sequence TTAGGG and a suite of protein complexes, forming the protective endcaps on chromosomes that shorten each time a cell divides (Blackburn 1991). Studies across vertebrate taxa have shown that while telomeres tend to shorten in relation to chronological age in a population, there can be considerable variation in telomere length among individuals of the same age (Dunshea et al. 2011). This variation, reflecting biological age, is thought to be mediated by a range of factors including inherited telomere length (Dugdale and Richardson 2018), environmental conditions during early life (McLennan et al. 2016; Dugdale and Richardson 2018), and extrinsic pressures throughout life, such as habitat (Ibáñez-Álamo et al. 2018), stress (Haussmann and Marchetto 2010), and disease (Beirne et al. 2014; Hammers et al. 2015). Declines in telomere length can be countered by telomere repair mechanisms, such as telomerase. Other telomere maintenance genes are still being discovered and seem to vary among tissues, individuals, and species (Dunshea et al. 2011; Foley et al. 2018).

Telomeres are present in all eukaryotic cells (Blackburn 1991), but the choice of tissue and the method of collection for the objective of age determination depend upon the study organism. In light of the impacts of white-nose syndrome on bat populations, non-lethal tissue sampling is preferred. This limits the types of tissue that can be collected, but permits the release of an organism after processing, with the potential for recapture

and carrying out longitudinal studies. A small biopsy of wing tissue, which heals in 2–3 weeks (Weaver et al. 2009; Greville et al. 2018), has been the standard for non-lethal obtention of DNA from bats, but other sources increasingly are being used. Mucosal epithelial cells, collected with buccal swabs, and gastrointestinal epithelial cells, found in guano, yield lower amounts of DNA than do wing biopsies (Corthals et al. 2015), but have been used successfully for genotyping (Puechmaille et al. 2007; Ramón-Laca et al. 2015; Oyler-McCance et al. 2018) and species identification (Walker et al. 2016) in bats. While buccal swabs have previously been used to study telomere length in edible dormice (*Glis glis*—Hoelzl et al. 2016b) and humans (e.g., Thomas et al. 2008; Finnicum et al. 2017), to our knowledge, guano or feces have not yet been used in any telomere studies.

The type of tissue used in studies of telomere dynamics may be important because telomeres may be shortened by different rates of cell division across tissue types, damaged by different levels of oxidative stress (von Zglinicki 2002), or repaired through differentially expressed mechanisms, such as telomerase (Gomes et al. 2011; Nussey et al. 2014; Foley et al. 2018). This results in disparities in telomere length and telomere attrition rate among tissue types. Numerous studies in humans have shown that telomere length and attrition rate are correlated among tissue types (Daniali et al. 2013; Schmidt et al. 2016; Finnicum et al. 2017), but results from other vertebrate species have been mixed (Reichert et al. 2013). Tissue samples collected non-lethally from bats, such as wing biopsies, buccal swabs, and guano, each contain epithelial cells, but experience different stressors and may, therefore, show differences in telomere length. Mucosal and gastrointestinal epithelial cells proliferate rapidly and experience high turnover rates due to their function as barriers from bacterial and mechanical damage during mastication and digestion (Squier and Kremer 2001), whereas skin epithelial cells typically divide slowly (Tian et al. 2018). For North American bats, however, the skin cells on their wings may be dividing at a faster rate than normal if they sustain wing damage (Cryan et al. 2010) due to *Pseudogymnoascus destructans* (Pd), the fungus that causes white-nose syndrome (Lorch et al. 2011).

The inability to age adult bats presents a challenge to describing age-related aspects of their biology, including demographic traits, with implications for the conservation of species impacted by white-nose syndrome. In this study, we investigated relative telomere length (RTL) as a possible marker of chronological age in two long-lived bat species, little and big brown bats. In addition, we explored RTL correlations and differences among tissue types collected from little brown bats and using longitudinal samples, we examined RTL as an indicator of dynamically changing ecological events that may impact biological age. Furthermore, because disease is known to influence telomere length in other taxa, we investigated whether infection from white-nose syndrome was associated with shorter telomeres in little brown bats, a highly impacted species (Frick et al. 2010).

MATERIALS AND METHODS

Study area and sample collection.—We sampled little brown bats at maternity colonies ($n = 6$) in Massachusetts, New Hampshire, and Vermont, from mid-May to mid-September of 2016–2019. Bats in all six colonies roosted in large wooden barns located within a 100 km radius centered in southwestern New Hampshire. Bats were banded beginning as early as 2006 at four sites and beginning in 2016 at two sites. We used harp traps (Bat Conservation & Management, Carlisle, Pennsylvania) to capture bats upon their return to the roost after their first foraging bout. All individuals were held in separate mesh bags before being identified to species, examined to determine sex and reproductive condition (Racey 2009), and measured for mass (g) and right forearm length (mm). We determined age (juvenile or adult) based on the presence of an epiphyseal gap in the fourth metacarpal joint (Kunz and Anthony 1982). For bats banded as adults and recaptured, we recorded their minimum age as years since initial capture plus one, such that if they were banded 5 years before recapture, they were at least 6 years old. Only bats banded as juveniles were considered to be known-age individuals. Wing damage associated with white-nose syndrome was assessed by transilluminating the wings over a light box and quantified using a modified version of a scoring system that ranks bats from zero (no damage) to three (heavy damage—Reichard and Kunz 2009). In addition, we collected wing swabs from a subset of bats to test for Pd and categorized them as positive or negative for the fungus based on assay results following the qPCR methods of Muller et al. (2013) and the sampling and analysis of Langwig et al. (2015). One wing biopsy was collected from a subset of bats captured on a given night using a sterile 2 mm (2016) or 3 mm (2017–2019) biopsy punch after cleaning the wing membrane with an isopropyl alcohol wipe. We also collected buccal cells from some bats by swabbing the inside of the mouth for one minute with a Whatman OmniSwab (GE Healthcare, Chicago, Illinois). Guano was collected opportunistically directly from the bats or from their holding bags. All samples were stored in separate tubes containing 0.5 ml RNAlater (Ambion, Austin, Texas) at -80°C until DNA could be isolated.

We collected big brown bats from maternity colonies ($n = 10$) roosting in buildings in and near Fort Collins, Colorado from 2005 to 2015. Study area descriptions in relation to use by big brown bats have been described in detail elsewhere (e.g., [Neubaum et al. 2007](#); [O'Shea et al. 2011](#)). During 2001–2005, we used mist nets, harp traps, funnel traps, and handheld nets to capture bats as they emerged from roosts around dusk. At capture, bats were examined to determine sex and reproductive condition, classified as adults or juveniles based on the presence of an epiphyseal gap, and implanted with PIT tags (AVID, Norco, California). Beginning in 2005 and opportunistically through 2015, previously tagged bats were removed from the population and euthanized in the course of other studies ([Cryan et al. 2012](#); [Castle et al. 2015](#)). All whole bats were stored at -80°C . In 2010, we collected two wing biopsies (3 mm) each from a subset of frozen bats and stored them in lysis buffer at room temperature until DNA extraction in 2019. To further

expand the age range of samples, wing biopsies were obtained in 2019 from the carcasses of six older bats that had been previously collected during 2010–2015; these tissues were stored briefly in RNAlater until DNA extraction. All big brown bats included in this study were unaffected by white-nose syndrome because Pd had not reached Colorado in 2015 (Neubaum 2018).

Handling and sampling protocols for little brown bats were approved by the University of New Hampshire's Institutional Animal Care and Use Committee and work was conducted under appropriate state permits from New Hampshire, Vermont, and Massachusetts. All capture, tagging, sampling, and euthanasia procedures for big brown bats were approved by the Institutional Animal Care and Use Committees of the U.S. Geological Survey and Colorado State University. Big brown bats were captured under authority of scientific collecting licenses issued by the Colorado Division of Wildlife. All procedures followed the guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes et al. 2011, 2016).

DNA isolation and estimation of relative telomere length.—We extracted genomic DNA from wing biopsy and buccal swab samples using the DNeasy Blood & Tissue kit (Qiagen, Inc., Valencia, California) following the Animal Tissue Spin-Column protocol. For buccal samples, we replaced the RNAlater with 1X Tris-EDTA after centrifuging and soaked swabs for 1 h prior to extraction to remove some of the RNAlater salts (Walker et al. 2016). Biopsy samples were lysed for at least 4 h and buccal swabs for at least 12 h. From guano samples, we extracted DNA using the DNeasy PowerSoil kit (Qiagen, Inc., Valencia, California) following the manufacturer's recommended protocols. We quantified DNA concentration of biopsy and guano samples using a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, California). All isolated DNA was used immediately or stored at -20°C or -80°C until further use.

We determined RTL following the method of Cawthon (2002), which estimates the ratio of telomere repeats to a single or non-variable copy number reference gene in a sample relative to a calibrator. The tel1b (CGGTTTGGTTTGGGTTTG GTTTGGGTTTGGGTTTGGGT) and tel2b (GGCTTGCCT TACCCTTACCCTTACCCTTACCCTTACCCT) primers were used for the telomere reactions (Callicott and Womack 2006). Following Smith et al. (2011), we selected the reference gene primers from a panel including rag2 (Corthals et al. 2015), 36B4 (Cawthon 2002), 36B4u (Callicott and Womack 2006), c-myc (Hoelzl et al. 2016a), and BDNF (Foley et al. 2018). We chose the primers developed based on chiropteran sequences (Corthals et al. 2015), rag2-q2-F1 (ACACCAAACAATGAGCTTTC) and rag2-q2-R1 (CCATATCTGGCTTCAGG), because they showed the most consistent amplification and cleanest melt curves. After selection of the primers, we ran a conventional PCR and gel electrophoresis to verify correct band size of amplicons for each sample type.

All qPCR reactions were carried out in 20 μ l final volumes consisting of 10 μ l 2X Quantifast SYBR Green Mastermix (Qiagen, Inc., Valencia, California), 4 μ l ultrapure water, 2 μ l of forward and reverse primers (1 μ M final concentration), and

2 µl of template DNA, with the exception of buccal samples, for which 4 µl of template DNA and 2 µl of water were used to increase the final DNA concentration in each well. No-template controls and a calibrator sample were run in triplicate on each plate. The calibrator sample for little brown bats came from a single individual not included in the analysis. For big brown bats, we pooled equal volumes of DNA eluate from 64 samples to create a calibrator sample. Samples were run in triplicate for both primer sets on the same plate to minimize inter-plate variation. Assays were run on a 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California) with the following thermocycling conditions: 95°C for 5 min, 40 cycles of 95°C for 10 s and 60°C for 30 s, followed by a melt curve at the end of each run. To assess inter-run repeatability, we repeated the analysis of a single plate of samples under the same conditions.

We analyzed raw, non-baseline corrected fluorescence data using LinRegPCR (Ruijter et al. 2009) with separate windows-of-linearity for each amplicon group (telomere and rag2). Minor adjustments were made to individual windows-of-linearity to improve correlations between points in the exponential phase of the amplification curves. We calculated coefficients of variation (CV) using the C_q values of sample replicates and where CV exceeded 5%, we excluded single replicates from further analysis when they were clear outliers (Ehrlich et al. 2009). The following equation was then used to calculate RTL:

$$\text{RTL} = \frac{E_T^{(C_{qT} \text{ calibrator} - C_{qT} \text{ sample})}}{E_S^{(C_{qS} \text{ calibrator} - C_{qS} \text{ sample})}}$$

where E_T and E_S are the mean primer efficiencies among all samples on a plate for the telomere (T) and rag2 (S) primers, respectively. Mean calibrator C_q values ($C_{qT \text{ calibrator}}$ and $C_{qS \text{ calibrator}}$) are used to standardize RTL values across plates compared to the mean C_q values ($C_{qT \text{ sample}}$ and $C_{qS \text{ sample}}$) for each sample (Pfaffl 2001).

Statistical analyses.—We analyzed RTL from the two species separately using R v3.6.1 (R Core Team 2018) and the “lme4” package (Bates et al. 2015). For big brown bats, we first considered linear mixed models with RTL as the response variable and age as the fixed effect. Reproductive status was excluded from the analysis because incomplete data would have biased the results. We did not include plate or year as random effects because bats were not randomly distributed among qPCR plates by age and year was highly correlated with age due to our sampling method. Including colony as a random effect did not improve model fit based on a likelihood ratio test (LRT; $P = 0.999$), multiple linear regression therefore was used for further analyses. We created a set of models to assess whether RTL was related to age, including linear, polynomial (quadratic and cubic), and null age terms, then selected the best model using Akaike’s Information Criterion corrected for small sample size (AICc). Residuals were plotted against fitted values and examined for homogeneity and normality to assess model fit.

All models for little brown bats used RTL from the wing biopsy samples as the response variable and included individual ID and year as random effects. When tested as a random effect, we found that colony did not improve model fit ($P = 0.257$) and was subsequently excluded from the analysis. We did not include qPCR plate as a random effect because bats were not randomly distributed among plates by age. An a priori set of models was created with biologically relevant combinations of age (linear and quadratic), wing score, and reproductive status as fixed effects. Julian date was not included as it was highly correlated with reproductive status and wing score. Age also was partitioned into within- and between-subject effects using within-subject centering, which can be used to distinguish individual variation in RTL measurements from population-level variation in RTL (van de Pol and Wright 2009). For bats sampled more than once, mean age between sampling points (between-subject) and delta age or age at sample minus mean age (within-subject) were calculated. For bats sampled once, mean age is simply age at sampling and delta age is zero. The best model was selected based on AICc and model fit was evaluated by examining the residuals. Likelihood ratio tests were used to compare the fit of nested models. Pd status (positive or negative) was evaluated in a separate linear mixed model because not all bats were tested for Pd. The best model for each species was run again with only bats of known age (tagged or banded as juveniles) to evaluate the influence of including individuals of unknown age on the models.

To examine drivers of change in RTL over time for little brown bats, individuals sampled in consecutive years ($n = 16$) were included in a separate longitudinal analysis. Multiple linear regression models were fit with delta RTL (change in RTL between years) as the response variable and initial reproductive status, wing score, and age included as fixed effects in separate models due to small sample size. Initial RTL was included as a fixed effect in each model to account for regression to the mean (Hoelzl et al. 2016a). Models were compared to a model with only initial RTL using likelihood ratio tests. To further explore within-subject changes in RTL, temporal autocorrelation was examined by fitting a linear mixed model of RTL_{T+1} with age and RTL_T as fixed effects and sample year as a random effect. The slope of the RTL_T term estimates the degree of temporal autocorrelation corrected for age effects (Fairlie et al. 2016).

Differences in RTL values among little brown bat tissues (biopsy, buccal, and guano) were examined using a linear mixed model with tissue type and age as fixed effects and individual ID as a random effect to control for multiple tissues coming from a single bat (although all three tissues were not collected from every individual). Differences in mean RTL among tissue types were determined post hoc with Tukey’s contrasts using the “multcomp” R package (Hothorn et al. 2008). Correlations in RTL among tissue types were determined using Pearson’s correlation tests. The same calibrator sample was used for all tissue types to allow for comparison.

RESULTS

We analyzed 153 wing biopsy samples from 122 female little brown bats ranging in age from 0 to ≥ 12 years old, 32 of which were banded as juveniles. Thirty-one little brown bats were sampled twice over the study period with time between sampling ranging from zero to three years. We also analyzed 34 buccal swabs and 32 guano samples collected from little brown bats. For big brown bats, we analyzed 73 wing biopsy samples from 71 females and 2 males ranging in age from 0 to ≥ 14 years old, 49 of which were PIT-tagged as juveniles.

The mean PCR efficiency for little brown bat samples was 1.93 ± 0.02 SD and 1.92 ± 0.02 for telomeres and rag2, respectively, while for big brown bats, PCR efficiency was 1.88 ± 0.02 for telomeres and 1.90 ± 0.01 for rag2. Mean inter-assay CV, based on the Cq values of the little brown bat calibrator samples, was 1.65% for both amplicons, while mean intra-assay CV was 1.88% for telomeres and 1.64% for rag2. For big brown bats, inter-assay CV was 2.54% for telomeres and 2.55% for rag2, while mean intra-assay CV was 2.44% for telomeres and 2.21% for rag2. RTL values from the samples analyzed twice were highly correlated ($R^2 = 0.89$, $P < 0.001$), supporting low among-run variation (Supplementary Data SD1).

RTL and predictor variables.—The best model for big brown bats indicated a quadratic relationship between RTL and age, although it only explained a small amount of the variation in RTL (adj. $R^2 = 0.24$, $F_{2,70} = 12.62$, $P < 0.001$). There also was support for a model with cubic age ($\Delta\text{AICc} = 0.79$), but the quadratic model was selected as the more parsimonious of the two (Table 1). RTL in big brown bat samples appeared to increase with age until approximately 4–8 years of age, then decrease (Fig. 1A). Results were similar when the quadratic model was run again with only known-age bats tagged as juveniles (adj. $R^2 = 0.14$, $F_{2,46} = 4.99$, $P < 0.05$).

Delta age and mean age were not significant in explaining variation in RTL in little brown bats (see below); we therefore used age and age² in the final models. The best model indicated a relationship between RTL and wing score (LRT, $\chi^2 = 4.720$, $P < 0.05$; Table 2), followed by models with age² ($\Delta\text{AICc} = 2.12$) and wing score and age ($\Delta\text{AICc} = 2.12$). Age by itself was not related to RTL ($\chi^2 = 0.109$, $P = 0.74$; Fig. 1B). We found similar results when the best model was run again with known-age bats only, but as a linear model due to small sample size (adj. $R^2 = 0.12$, $F_{1,33} = 5.56$, $P < 0.05$). Upon closer inspection of our data, we determined that, by chance, individuals with higher wing scores all were 1- or 2-year-old bats, which was

not representative of our sampling population. To test whether the uneven distribution of wing scores by age influenced our results, we reran the model with only the 1- and 2-year-old bats and observed the same effect of wing score on RTL, while the inclusion of age had less support (Supplementary Data SD2). Tukey's post hoc tests showed that bats with more wing damage (WS = 2) had significantly shorter telomeres than those with a wing score of zero ($P < 0.05$), but not than those with intermediate wing damage ($P = 0.12$; Fig. 2A). Bats with a wing score of zero or one had similar telomere lengths ($P = 0.32$). We also found significant support for Pd status influencing telomere length compared to a null model ($\chi^2 = 5.245$, $P < 0.05$; Table 3), where Pd-positive individuals ($n = 45$) had significantly shorter telomeres than Pd-negative ($n = 50$) individuals (Tukey, $P < 0.05$; Fig. 2B), independent of age ($\chi^2 = 0.206$, $P = 0.65$).

Longitudinal samples and tissue comparisons.—We detected a significant, but weak between-individual effect of age within little brown bats sampled twice (LRT, $\chi^2 = 4.388$, $P < 0.05$); however, when all bats were included in the analysis, we found no significant within- ($\chi^2 = 0.067$, $P = 0.79$) or between-individual ($\chi^2 = 0.637$, $P = 0.43$) effects of age on RTL. It is possible that RTL of bats sampled once may have masked any within- or between-subject effects in the dataset because there were nearly three times more bats sampled once than twice. Initial reproductive status ($t = 0.739$, $P = 0.48$), wing damage ($t = -0.983$, $P = 0.34$), and age ($t = -0.181$, $P = 0.86$) were not significant in explaining changes in RTL across ages in bats sampled in consecutive years ($n = 16$) when accounting for regression to the mean. In addition, there was no significant temporal autocorrelation in RTL measurements of individuals between years ($\chi^2 = 1.134$, $P = 0.29$). Changes in telomere length within individuals appeared to be complex (Fig. 3) and were not explained by any covariates that we collected on little brown bats.

We found no correlation in RTL between wing tissue and buccal cells within individual little brown bats ($R = 0.01$, $P = 0.98$, $n = 32$). Similarly, guano RTL was not correlated with RTL in wing tissue ($R = -0.05$, $P = 0.79$, $n = 29$) or buccal cells ($R = 0.11$, $P = 0.60$, $n = 25$; Fig. 4). Mean wing tissue and buccal cell RTL measurements were not significantly different (Tukey; $P = 0.99$), but RTL was significantly longer in guano than in wing tissue ($P < 0.001$) and buccal cells ($P < 0.001$; Fig. 5), independent of age (LRT, $\chi^2 = 0.746$, $P = 0.39$). Guano samples had higher variation in RTL (mean $\pm$ SD: 2.57 ± 1.92) than wing tissue (1.14 ± 0.31) and buccal swabs (1.13 ± 0.62).

Table 1.—Summary of multiple linear regression models of relative telomere length and chronological age in big brown bats (*Eptesicus fuscus*) sampled in and near Fort Collins, Colorado, 2005–2015. Linear, quadratic, cubic, and null regression models were considered and ranked by AICc. Coefficient estimates are shown for each model. K = number of parameters; logLik = log likelihood; w_i = model weight.

Model	Intercept	Age	Age ²	Age ³	K	logLik	AICc	ΔAICc	w_i
Age ²	0.926	0.136	−0.012		3	−17.47	43.52	0.00	0.60
Age ³	0.989	0.053	0.007	−0.001	4	−16.71	44.31	0.79	0.40
Null	1.165				1	−28.71	61.58	18.06	0.00
Age	1.203	−0.008			2	−28.47	63.29	19.76	0.00

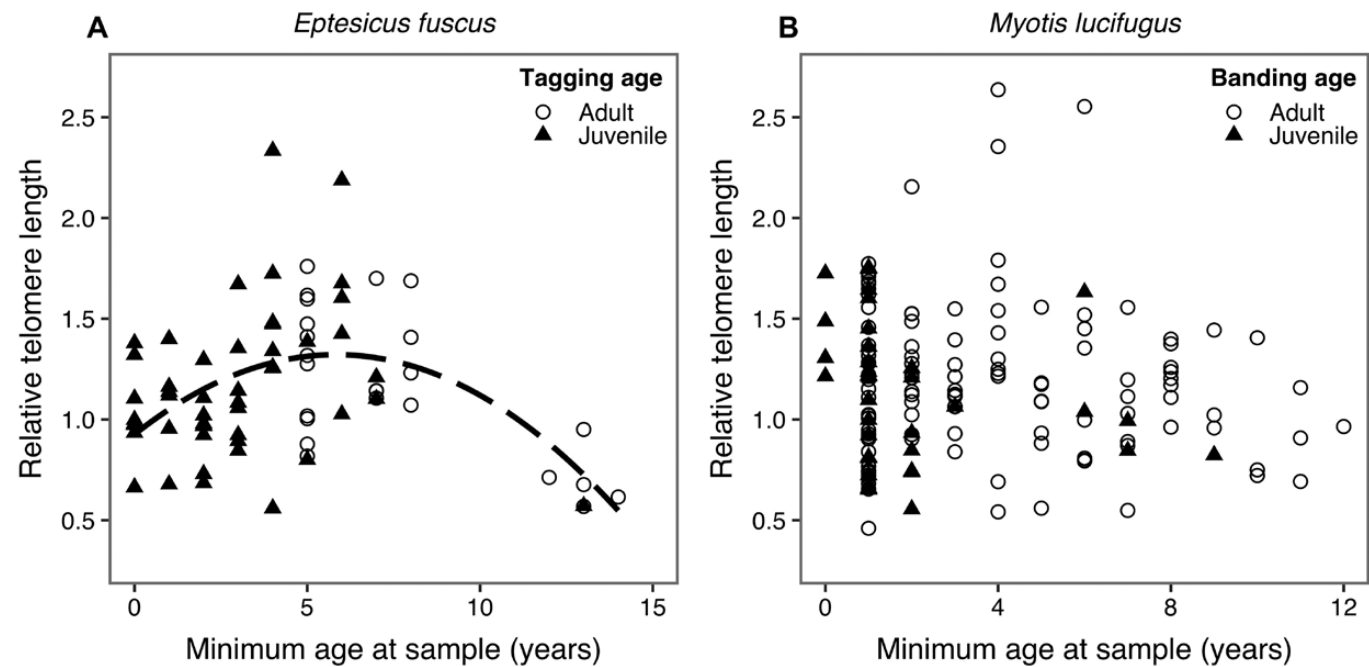


Fig. 1.—The relationship between relative telomere length and age in (A) PIT-tagged big brown bats (*Eptesicus fuscus*) sampled in and near Fort Collins, Colorado, 2005–2015 and (B) banded little brown bats (*Myotis lucifugus*) sampled in New England, 2016–2019. Bats marked as juveniles (closed triangles; known-age) and adults (open circles; minimum known-age) were combined in the model analysis. The dashed black line indicates a best-fit regression through the data where significant.

Table 2.—Summary of linear mixed models of relative telomere length and predictor variables in little brown bats (*Myotis lucifugus*) sampled in New England, 2016–2019. Linear and quadratic age terms were considered along with wing score (WS) and reproductive status (Rep) at the time of capture. Models were ranked by AICc. Coefficient estimates are shown for each model. K = number of parameters; logLik = log likelihood, w_i = model weight.

Model	Intercept	Age	Age ²	WS	Rep	K	logLik	AICc	ΔAICc	w_i
WS	1.243			−0.103		4	−58.23	126.88	0.00	0.42
Age ²	1.140	0.083	−0.008			5	−58.21	129.00	2.12	0.14
Age + WS	1.069	−0.002		−0.106		5	−58.21	129.00	2.12	0.14
Null	1.191					3	−60.59	129.46	2.58	0.12
Rep	1.234				+	7	−56.65	130.30	3.42	0.08
Age	1.198	0.004				4	−60.54	131.49	4.61	0.04
Rep + WS	1.240			−0.037	+	8	−56.45	132.16	5.28	0.03
Age + Rep	1.234	−0.001			+	8	−56.65	132.55	5.68	0.02
Age + Rep + WS	1.288	−0.001		−0.038	+	9	−56.45	134.44	7.57	0.01

DISCUSSION

Our results demonstrate that telomere length is not a reliable predictor of chronological age in little or big brown bats and that age-related declines in telomere length vary between the two bat species we studied. While we did not find a correlation between age and RTL in little brown bats (Fig. 2B), we detected a quadratic relationship between age and RTL in big brown bats (Fig. 2A), albeit one with little predictive power to age individuals of unknown ages. Our results are similar to those of Foley et al. (2018), who did not find a correlation between RTL and age in *M. myotis* and *M. bechsteinii*, but found linear relationships in *Rhinolophus ferrumequinum* and *Miniopterus schreibersii*. This suggests that there may be something unique about age effects on telomeres of *Myotis* spp. Differences among species, particularly *Myotis* spp. compared

to other genera, are potentially due to differences in telomere maintenance strategies. Some species of bats suppress or limit expression of telomerase (Gomes et al. 2011; Foley et al. 2018), similar to large-bodied mammals (Tian et al. 2018), and instead rely on other mechanisms for DNA maintenance. Several recent studies have suggested a relationship between longevity and the unique set of genes under positive selection in *Myotis* spp., including those involved in repairing DNA, resisting tumors, and reducing oxidative damage (Ma et al. 2016; Tian et al. 2017; Foley et al. 2018). In *M. lucifugus*, the telomere maintenance genes *DKC1* and *TERT* are under positive selection (Morgan et al. 2013). In addition, certain telomere maintenance genes appear to be differentially expressed in *Myotis* spp. compared to other mammals (Foley et al. 2018). This may explain the lack of telomere attrition observed in little brown bats, but further

work is needed in non-*Myotis* species to see if there are phylogenetic patterns in telomere length maintenance. There also is room for more study within the genus, as the three *Myotis* species studied thus far all fall into lineages that have high maximum longevity according to current lifespan data (Wilkinson and Adams 2019).

The quadratic relationship between age and RTL in big brown bats has been observed previously in several vertebrate taxa, including mammals, reptiles, and fish (Anchelin et al. 2011; Fairlie et al. 2016; Rollings et al. 2017). Increases in RTL for middle-age individuals have been explained in part by selective disappearance, or mortality of young individuals with shorter telomeres (Fairlie et al. 2016), and partly by within-individual increases in telomere length, which could be due to increased telomerase expression (Ujvari et al. 2017). Similar to little brown bats, big brown bats appear to suppress telomerase (Gomes et al. 2011) and may instead have evolved alternate mechanisms for telomere maintenance. Both species

are hibernators with high maximum longevity (Wilkinson and Adams 2019) and appear to be able to reduce oxidative damage compared to other groups of mammals of similar size or with similar metabolic rates (Brunet-Rossinni 2004; Brown et al. 2009). Because our study did not allow for a longitudinal study of big brown bats, we cannot distinguish between selective disappearance and within-individual changes in telomere length as potential explanations for the observed quadratic trend.

Although our cross-sectional data show no significant changes in RTL for little brown bats with age, the longitudinal data suggest a more complex pattern of individual increases and decreases in RTL between years. Similar longitudinal patterns can be seen in Soay sheep (*Ovis aries*—Fairlie et al. 2016); European badgers (*Meles meles*—van Lieshout et al. 2019); Seychelles warblers (*Acrocephalus sechellensis*—Spurgin et al. 2017); great tits (*Parus major*—Salmón et al. 2017); and frillneck lizards (*Chlamydosaurus kingii*—Ujvari et al. 2017). Despite concerns of measurement error (Steenstrup et al. 2013), a recent study (Bateson and Nettle 2017) as well as our low inter- and intra-plate CVs and high inter-plate repeatability (Supplementary Data SD1) suggest that many of the observed changes in RTL were real. The lack of temporal autocorrelation between RTL measurements among years as well as the lack of support for within-subject age-related declines in RTL also support the complex patterns in telomere length we observed in little brown bats (Fig. 3).

While we were unable to explain inter-annual changes in RTL with reproductive status and age, there was evidence that white-nose syndrome was associated with shorter telomeres in little brown bats. Infections have been associated with shorter telomere length and poor biological state in several species (Monaghan 2010), including malaria in birds (Asghar et al. 2015; Hammers et al. 2015) and humans (Asghar et al. 2018); bovine tuberculosis in European badgers (Beirne et al. 2014); and *Salmonella enterica* in experimentally infected house mice (*Mus musculus musculus*—Ilmonen et al. 2008). Hibernating bats in North America can be repeatedly infected with Pd each winter when they pick up the fungus from the substrate of hibernacula and from other bats (Lindner et al. 2011; Lorch et al. 2013). Pd infection loads gradually increase over the winter (Langwig et al. 2015) and cause changes in hibernation patterns. Colonies undergoing the early stages of WNS invasion show increased arousal frequency (Reeder et al. 2012), while remnant colonies that have been exposed to Pd for a decade or more appear to have similar arousal rates to pre-WNS bats, but arouse for longer periods and from lower skin temperatures (Lilley et al. 2016). Both increased arousal frequency and time

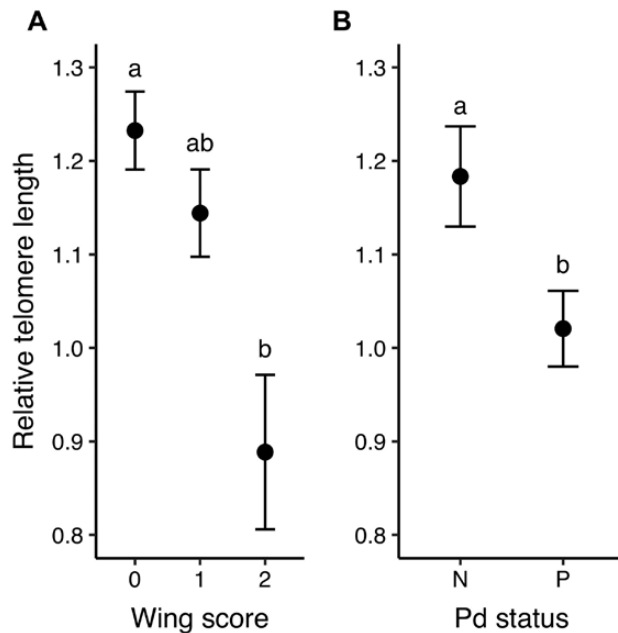


Fig. 2.—The relationship between little brown bat (*Myotis lucifugus*) relative telomere length and (A) wing score, where 0 indicates no damage and 2 indicates moderate to high levels of wing damage, and (B) Pd status for individuals that tested negative (N) or positive (P) for *Pseudogymnoascus destructans* (Pd), the fungus that causes white-nose syndrome in bats. Bats were captured in New England, 2016–2019. Points represent mean RTL and bars represent $\pm$ standard error. Letters indicate significantly different means among groups.

Table 3.—Summary of linear mixed models of relative telomere length and predictor variables in little brown bats (*Myotis lucifugus*) sampled in New England, 2016–2019, and tested for *Pseudogymnoascus destructans* (Pd). Models were ranked by AICc. Coefficient estimates are shown for each model. Pd = Pd status (positive or negative) at time of capture; K = number of parameters; logLik = log likelihood, w_i = model weight.

Model	Intercept	Pd	Age	K	logLik	AICc	Δ AICc	w_i
Pd	1.180	+		3	-29.13	68.93	0.00	0.61
Pd + Age	1.202	+	-0.006	4	-29.03	71.00	2.07	0.22
Null	1.103			2	-31.75	71.95	3.02	0.13
Age	1.115		-0.003	3	-31.72	74.12	5.19	0.05

spent euthermic have been associated with higher levels of telomere damage in edible dormice (Hoelzl et al. 2016a). Although bats naturally arouse periodically during the winter, it appears that Pd infection may contribute to telomere damage as a result of altered hibernation patterns, leading to bats having shorter telomeres when they emerge from hibernation.

For individuals that survive the winter, Pd infection results in a flare-up of wing damage within a few weeks after emergence from hibernation (Fuller et al. 2011; Meteyer et al. 2011; Fuller 2016). In the spring, females are recovering from hibernation, migrating to summer sites, and initiating fetal development, all energetically costly activities that compound the added costs of fighting Pd infection and recovering from WNS (Moore et al.

2013; Fuller 2016). Given these added costs, it is unsurprising that infected bats have shorter telomeres; however, it also is possible that bats can recover and restore telomere length over the course of the summer (Fig. 3). Djungarian hamsters (*Phodopus sungorus*) that use spontaneous daily torpor are able to elongate their telomeres and recover from telomere losses sustained during hibernation (Turbill et al. 2012). Bats also use torpor during the active season, particularly as an energy-saving strategy in the spring and summer, to compensate for cold temperatures and low food availability (Racey 1973; Besler and Broders 2019), but potentially also for telomere maintenance. Torpor may allow bats to repair their telomeres and even compensate for increased cell turnover in portions of their wings that are damaged repeatedly by white-nose syndrome. In a study of forced tissue regeneration via fin-clipping, telomere length remained stable in zebrafish (*Danio rerio*) over their lifetime (Lund et al. 2009), suggesting that some animals may have cellular mechanisms, such as those discussed above, capable of maintaining telomere length in individuals over time.

Our results suggest that wing biopsies currently are the best source of tissue for studying telomere dynamics in bats. Buccal RTL was not significantly correlated with wing biopsy RTL, unlike the results of Hoelzl et al. (2016b). We cannot, however, rule out the use of buccal swabs as an alternative to wing biopsies for studying telomere dynamics in bats when the qPCR method is used, which requires a small amount of DNA compared to other telomere measurement techniques (Nakagawa et al. 2004). Our data suggest that storage time may have affected RTL measurements for the buccal swabs, where swabs stored for longer periods of time had more variable RTL. Although we were able to amplify telomere sequences from guano samples, there was much higher variation in RTL among individuals and there was no correlation in guano RTL with either buccal or wing biopsy RTL (Figs. 4 and 5). Boston et al. (2012) successfully used wing tissue and guano samples for genotyping purposes, but it is possible that telomere length assessment is more susceptible to PCR inhibitors present in guano samples (Taberlet et al. 1999) or that telomeres are more

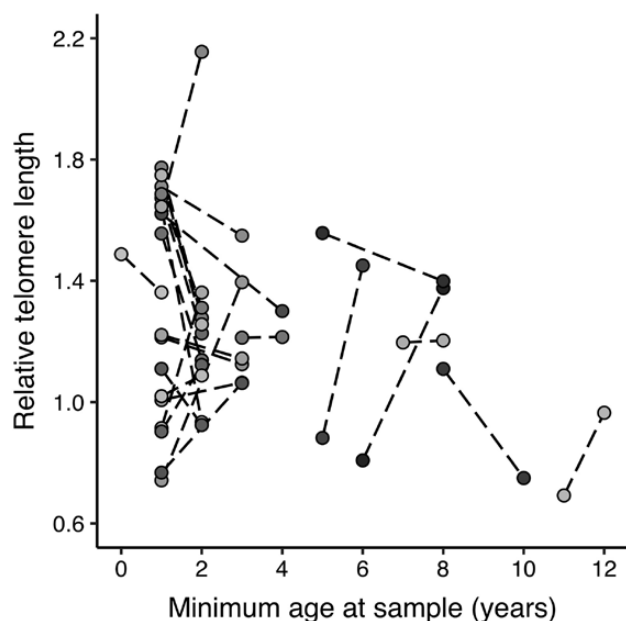


Fig. 3.—Changes in relative telomere length between years, with samples collected from individual little brown bats (*Myotis lucifugus*) connected by dashed lines. Bats shown here were sampled twice over intervals of one to three years in New England, 2016–2019.

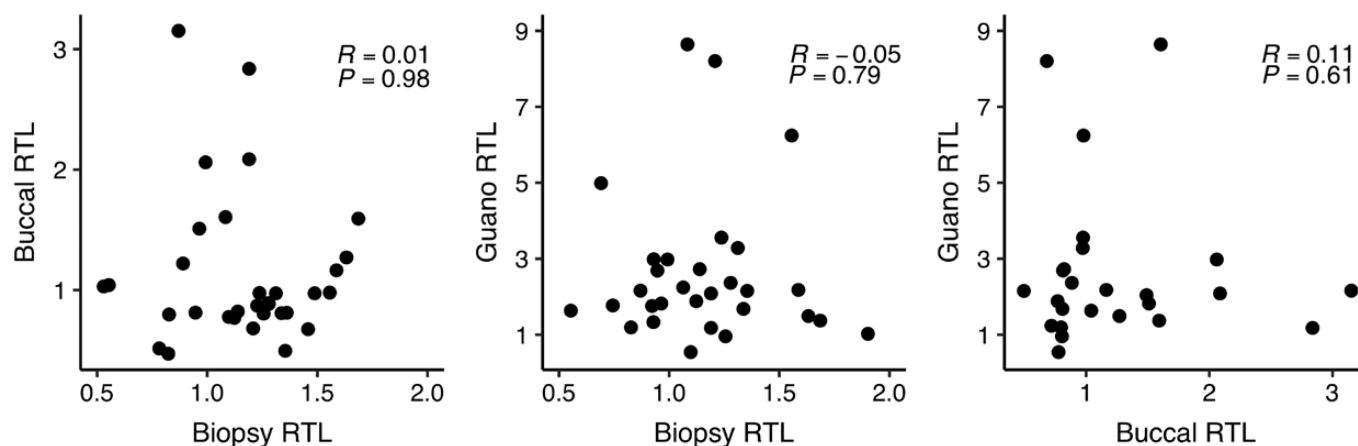


Fig. 4.—Correlation in relative telomere length (RTL) between wing (biopsy), mucosal epithelial (buccal), and gastrointestinal epithelial (guano) tissues collected from the same individual little brown bats (*Myotis lucifugus*) in New England, 2016–2019. All three tissue types were not collected from every individual. Note that scales differ between graphs.

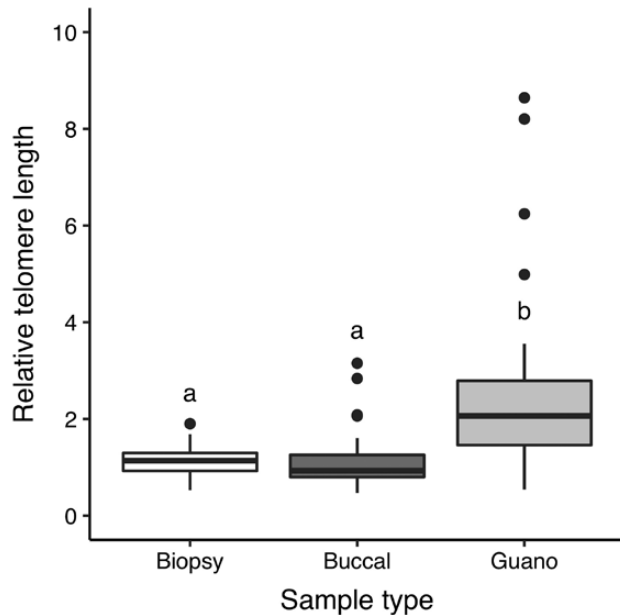


Fig. 5.—Boxplot showing relative telomere length variation within and among sample types. Wing (biopsy), mucosal epithelial (buccal), and gastrointestinal epithelial (guano) tissues were collected from the same individual little brown bats (*Myotis lucifugus*) in New England, 2016–2019. Letters indicate significantly different means among groups.

degraded in shed gastrointestinal epithelial cells. There also is much less endogenous DNA in guano relative to bacterial and insect DNA (Ramón-Laca et al. 2015); however, the telomere primers used in this study only should have amplified vertebrate telomeres (Hinnebusch et al. 1990; Vitková et al. 2005). Further refinement of sampling and assessment procedures may help reduce the variability in buccal swab and guano RTL measurements, making it easier to use these types of non-invasive samples in future telomere studies.

We found no significant difference between buccal and wing biopsy mean RTL, which was unexpected given the different turnover rates in these epithelial cells (Squier and Kremer 2001; Tian et al. 2018). Results from two human studies showed similar RTL between these tissue types, but were inconclusive (Gadalla et al. 2010; Dlouha et al. 2014). The variety of cell types found in each tissue may be partially obscuring differences in RTL. The wing membranes of bats are composed of epidermal epithelial, lymphatic, blood, nerve, and muscle cells (Cryan et al. 2010), whereas buccal samples can contain low levels of leukocytes (Finnicum et al. 2017; Theda et al. 2018). Multiple cell types in a sample may decrease observed differences in RTL between tissues even if RTL differs between cell types. Other studies that have compared tissue types suggest that telomere length differences are established during early life (Daniali et al. 2013; Reichert et al. 2013; Schmidt et al. 2016) and are not related to cell replication rates (Thomas et al. 2008). It also is possible that the mechanisms by which these tissues arrived at their current telomere lengths are drastically

different, resulting in observed similarities that mask underlying tissue-specific telomere dynamics.

Our findings demonstrate that RTL in little brown bats is more a reflection of biological age than chronological age. In this species, telomere length was influenced by infection and wing damage associated with white-nose syndrome, but not chronological age. In big brown bats, RTL was quadratically associated with age, but our model explained little of the variation in RTL. Reasons for differences in the patterns between the two species are as yet unclear. Although telomere length will most likely not be useful in determining chronological age of unknown-age bats, other molecular biomarkers such as DNA methylation patterns (De Paoli-Iseppi et al. 2017; Wright et al. 2018) may be more successful.

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SUPPLEMENTARY DATA

Supplementary data are available at *Journal of Mammalogy* online.

Supplementary Data SD1.—Plot showing repeatability in relative telomere length measurements between qPCR runs of identical samples from little brown bat wing tissue collected in New England, 2016–2019.

Supplementary Data SD2.—Summary of linear mixed models of relative telomere length and predictor variables in 1- and 2-year-old little brown bats sampled in New England, 2016–2019, with only 1- and 2-year-old bats included.

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