15

Methods for Age Estimation and the Study of Senescence in Bats

ANJA K. BRUNET-ROSSINNI
GERALD S. WILKINSON

OVER THE PAST SEVERAL DECADES, bat biologists have accumulated data on the lifespans of 65 species of bats. The current list of species-specific maximum longevities is surprisingly extensive (see Wilkinson and South, 2002, appendix; Gaisler et al., 2003), considering that these data are obtained from the fortuitous recapture of individuals that were tagged during their birth year. From these data, it is clear that bats are relatively long-lived mammals. On average, bats live three times longer than nonflying eutherian mammals of similar body size and metabolic rate (Austad and Fischer, 1991). While this extreme longevity poses intriguing questions for researchers interested in determinants of longevity and senescence, it also poses practical obstacles to the investigation of biological phenomena in which age is a critical factor. Determining the age of wild, untagged mammals can be difficult. This difficulty is particularly poignant in bats because with such extended longevities, it is likely that they experience more environmental and physiological variation (particularly that associated with age) than short-lived mammals and this variation may confound several aspects of bat biology that we aim to investigate.

In this chapter, we describe techniques for age determination and discuss senescence and longevity in bats. We present methods currently available for the determination of age in juvenile and adult bats, highlight the advantages and disadvantages of each, and provide guidelines to use when selecting a particular method. We also suggest new ideas, which, with proper research and the development of standards, may result in novel techniques that can overcome some of the limitations of current methods and thus enhance our ability to determine chronological and/or biological age of bats. Finally, we briefly summarize current research on bat longevity and senescence research that often requires knowledge of chronological age of individuals and present ideas for future investigations in this line of research.

—-1
—0
—+1

ESTIMATION OF CHRONOLOGICAL AND/OR BIOLOGICAL AGE

In this discussion, we make a distinction between chronological and biological age. Chronological age is the time interval between the present and the time of birth of an individual. Biological or physiological age reflects the life expectancy of an individual and is based on physical changes in the morphology and/or function of the body. It is easy to envision how the biological age of an individual may not always coincide with its chronological age. The only means of knowing the exact chronological age of a bat is to permanently mark it at birth using an appropriate method (see Kunz and Weise, this volume), so at any subsequent recaptures, the exact age of the bat can be determined. The drawbacks of this method for obtaining age information are that marking bats can be time consuming, it requires long-term monitoring, and recapture rates are typically low, not to mention that the process itself may disturb the bat. Given these limitations, bat researchers must decide whether their research requires knowledge of exact chronological age or whether an estimate based on biological age will suffice.

Several methods have been established for the determination of age in bats. Because they are all based on morphological traits, they provide estimates of chronological age from a reference standard by which chronological age can be correlated with biological age. Each of these methods has limitations, and unlike tagging individuals at birth, none results in knowledge of the exact chronological age of the individual. These limitations stem from a number of difficulties associated with determining age in bats and ultimately lead to decreased predictive power. To begin, once bats reach full adult size, they show few visible markers of age. Second, no method includes age determination of very old bats, likely because the oldest individuals are few and seldom captured. Third, no method can easily account for anatomical and morphological variation among individuals resulting in differences in growth patterns. These differences arise from genetic structuring of populations and environmental variation such as dietary differences, microenvironmental differences during development, timing of birth, etc. (e.g., Hoying and Kunz, 1998). Moreover, a reference standard for an age determination technique generated with a captive colony cannot be used with confidence for aging individuals in the wild because their growth patterns may differ (Kunz and Hood, 2000). Finally, methods that claim to provide the highest predictive power in adult bats, such as counting incremental lines of dentine and cementum, are invasive, require specific equipment that limits their utility in the field, and are of questionable accuracy (Phillips et al., 1982; Anthony, 1988).

Because the methods for one age category are seldom useful for the other, in this publication we classify the techniques currently available for age estimation of bats into those suitable for juveniles and for adults. We encourage readers interested in using any of the following techniques to have a clear idea of the level of accuracy and precision needed for age estimations and the logistical limitations of the study. Most importantly, researchers should consult the literature that originally presented these methods and reference standards. As guidelines, Anthony (1988) listed four questions that bat researchers should address when selecting an age estimation method. We reiterate these questions here:

1. Which age groups must be distinguished and how precise must these groups be? For some studies, placing individuals into broad, relative age groups, such as juvenile versus adult, may suffice. This categorization is easily accomplished by visualizing the level of ossification of bones in the wings (see epiphyseal-diaphyseal fusion method) and noting sexual traits such as the presence of teats and scrotal testes (see Racey, this volume). Other studies may be interested in more specific age categories that, for example, indicate the developmental stage of an individual or place adults into a specific age category.

2. Will live or dead specimens be evaluated, and will they be inspected repeatedly? If the research goals require the assessment of age on the same individuals over time, it will be essential to use age determination methods that do not harm the bats or impact their behavior or health. Using museum specimens makes it possible to use invasive methods of age estimation (such as incremental dentin and cementum lines), although researchers should keep in mind the need to preserve the integrity of specimens for future use (see Simmons and Voss, this volume).

3. Under what conditions will estimates be made, and what equipment will be available? Working in the field may require using a method that results in quick age estimations that rely on a minimum amount of equipment. Some methods, however, require specialized equipment and training that may limit their use to a laboratory setting.

4. Are data available regarding the use of a particular method of age estimation for a species or population of interest? The utility of a particular age estimation method for a particular population of bats may be influenced by geographic variation and environmental factors that impact patterns of postnatal growth. A reference standard developed for a specific population of bats may not necessarily apply to another population of the same species. It is imperative to assess the validity of the method for the particular study species based on existing data and knowledge of the population of interest. If a new reference standard is to be developed, the following are important criteria for the validity of the standard. First, the standard should be developed using individuals from the population of interest. Parry-Jones (2002) found a significant difference between the growth curves generated from length of forearm in captive versus wild *Pteropus poliocephalus*, al-

though other studies have found no differences (Kunz and Hood, 2000; Elangovan et al., 2002). Second, the reference standard should be generated with longitudinal sampling (mark-recapture) rather than cross-sectional sampling, as the latter significantly underestimates growth rates and thus the reliability of these data for age estimation (Baptista et al., 2000). Third, the standard should include known-age individuals spanning the entire lifespan of the species.

Existing Techniques for Estimating Age of Juveniles

Linear Growth of Long Bones

During the first number of weeks after birth, juvenile bats show a phase of linear growth of long bones. Length of forearm, metacarpals, and digits can be used to distinguish juveniles from adults, though length of forearm is the most commonly used character. With a species-specific reference standard that correlates these measurements with chronological age of known-age individuals, this method provides relatively accurate age estimates with 95% confidence intervals as small as ±1–2 days (Anthony, 1988). These reference standards have been developed for 49 different species, 42 of which are cited in Kunz and Hood (2000) and the others are *Hipposideros terasensis* (Cheng and Lee, 2002), *Rousettus leschenaulti* (Elangovan et al., 2002), *Pteropus poliocephalus* (Parry-Jones, 2002), *Megaderma lyra* (Rajan and Marimuthu, 1999), *Myotis daubentoni* (Richardson, 1990), *Myotis blythii* (Sharifi et al., 2002), and *Lasiurus cinereus* (Koehler and Barclay, 2000).

The advantages of this method are the accuracy of its predictive power, its ease of use in the field and laboratory, and its lack of invasiveness. Only a caliper or a ruler is needed to obtain repeatable measurements, making this method ideal for use on both live animals and museum specimens. The major disadvantage is that these long bones grow linearly only during the first few weeks of life. The time interval during which the above reference standards are valid ranges from 12 days in *M. lucifugus* to 45 days in *R. leschenaulti*. Other techniques are necessary to estimate the age of older individuals.

Epiphyseal-diaphyseal Fusion

After the initial linear growth phase of long bones, researchers can use changes in the patterns and rates of closure of the cartilaginous epiphyseal growth plates to estimate the age of juvenile bats. In its simplest form, this method can be used to qualitatively distinguish between young bats and adults. By transilluminating the wing of an individual using a headlight, a researcher can visualize the cartilaginous zone of the long phalanges because less mineralized tissue allows more light to pass through and thus appears lighter than bone. As the bat continues to grow, the epiphyseal plates eventually close until they are no longer visible to the unaided eye. In some species, the shape of the joints can be used to distinguish juveniles up to one year of age, as the phalangeal-metacarpal joints of juveniles are less knobby and more evenly tapered than those of adults. Used to this extent, this method requires no more than a headlight or flashlight and is ideal for distinguishing young of the year from adults in both the field and laboratory.

In its more sophisticated form, this method uses the total length of the cartilaginous region (total epiphyseal gap) between the bony diaphysis of a metacarpal and the bony diaphysis of the proximal phalanx to generate accurate quantitative estimates of age. This is the most commonly used method to estimate the age of juveniles beyond the age estimate provided by length of forearm. Total epiphyseal gap can be measured by transilluminating the wing placed on the stage of a dissecting microscope with a substage light source and using an ocular micrometer to measure the cartilaginous region. Some researchers have used calipers to measure the length of the total gap, but this method provides much less precision in measurement. Even further accuracy and precision of measurements can be achieved by using x-ray or other noninvasive digital tissue imaging technology to measure changes in the total epiphyseal gap, but the lack of portability generally prevents the use of this equipment in the field (Fig. 15.1).

Standard references have been developed using total gap of the fourth metacarpal-phalangeal joint of juvenile *Myotis lucifugus* (Kunz and Anthony, 1982), *E. fuscus* (Burnett and Kunz, 1982), *P. subflavus* (Hoying and Kunz, 1998), *H. terasensis* (Cheng and Lee, 2002), *M. myotis* (De Paz, 1986), *R. leschenaulti* (Elangovan et al., 2002), *P. mimus* (Isaac and Marimuthu, 1996), *P. hastatus* (Stern and Kunz, 1998), *T. brasiliensis* (Kunz and Robson, 1995), *P. poliocephalus* (Parry-Jones, 2002), *Megaderma lyra* (Rajan and Marimuthu, 1999), *M. daubentoni* (Richardson, 1990), and *M. blythii* (Sharifi et al., 2002).

Tracking the linear increase and subsequent linear decrease in total epiphyseal gap significantly extends the period of quantitative age estimation for juvenile bats. The predictive interval depends on species; for example, ages can be estimated up to 29 days in *M. lucifugus*, 75 days in *R. leschenaulti*, and 78 days in *M. myotis* (De Paz, 1986). Most reference standards for estimating age of juvenile bats use two regression equations. The first is based on length of forearm and is used to age juveniles during the early linear phase of growth. Thereafter, a second regression equation based on the rate of epiphyseal-diaphyseal fusion is used until the cartilaginous region becomes too small for visual observation. Alternatively, researchers could design an "ossification index" that combines characteristics of epiphyseal cartilage closure and measurement of long bones from museum specimens, as developed by Rybár (1969, 1971) for *M. myotis* and *Rhinolophus hipposideros*.

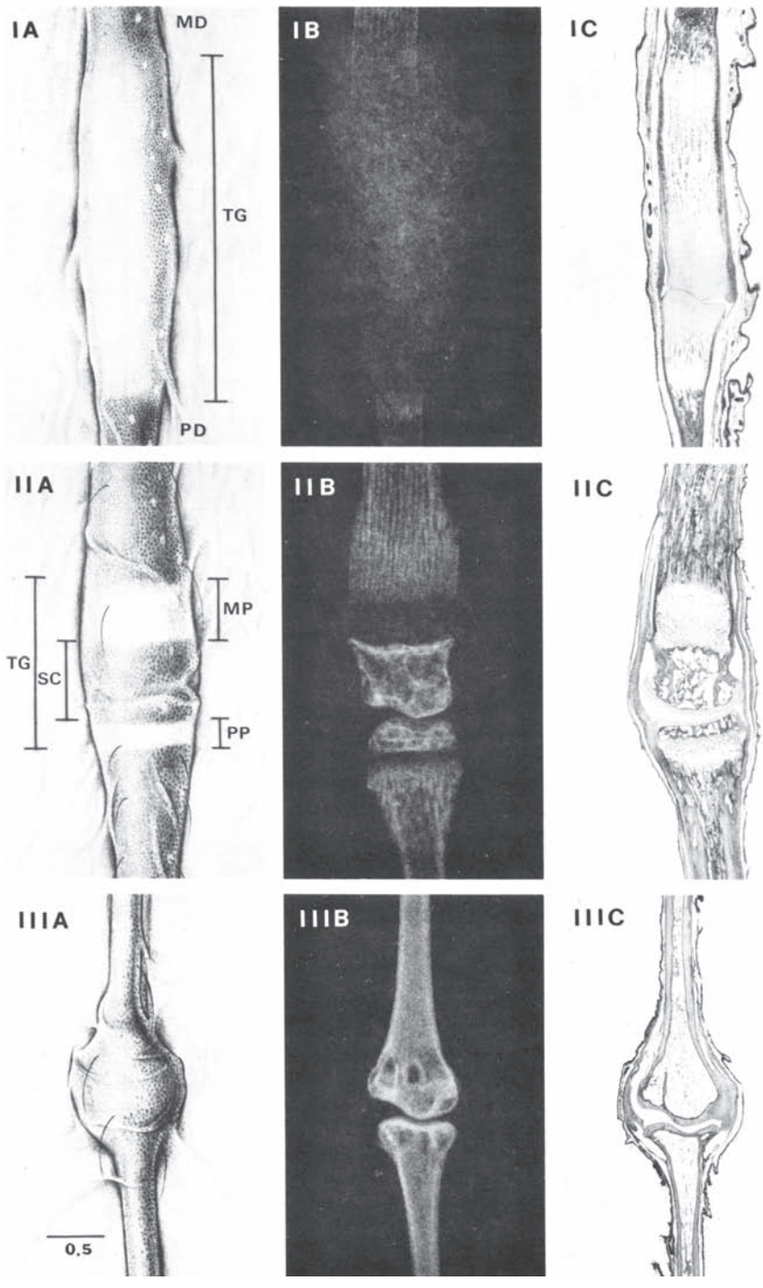

Figure 15.1. Growth progression in the metacarpal-phalangeal joint of *M. lucifugus* from a neonatal stage (I) to an adult stage (III), based on changes perceived in transilluminated wings (A), x-rays (B), and histological sections (C). Measurements taken on transilluminated joints are identified in I-A and II-A. Explanation of abbreviations: MD, metacarpal diaphysis; PD, phalangeal diaphysis; TG, total (epiphyseal) gap; MP, metacarpal epiphyseal plate; PP, phalangeal epiphyseal plate; SC, secondary center of ossification. Reference bar=0.5 mm.

Body Mass and Pelage Coloration

Body mass of juvenile bats increases linearly during early postnatal growth and generally can be used to estimate age, but because this trait is more variable than long bones during the postnatal growth period, it is a less reliable estimator of age (see Kunz, Adams and Hood, this volume). Notwithstanding, growth curves based on body mass have been described for *T. brasiliensis* (Kunz and Robson, 1995), *P. hastatus* (Stern and Kunz, 1998), *R. leschenaulti* (Elangovan, et al., 2002), *H. terasensis* (Cheng and Lee, 2002), *Megaderma lyra* (Rajan and Marimuthu, 1999), *P. mi-*

mus (Isaac and Marimuthu, 1996), and *P. subflavus* (Hoying and Kunz, 1998). Increase in body mass generally is linear until 2 to 7 weeks of age, depending on species. However, the predictive power of body mass for estimating age is limited by several factors such as genetic makeup, roost microenvironment, and food availability, each of which are known to influence the rate a which juveniles gain body mass (Kunz and Stern, 1995; Kunz and Hood, 2000; Parry-Jones, 2002). Most researchers prefer using changes in the long bone and the total epiphyseal gap length because of the variation associated with body mass and the limited time period over which body mass is useful for estimating age.

Pelage coloration often differs between juveniles and adults in many species and thus may provide important indices of age. This difference has been used to qualitatively distinguish between juveniles and adults (Anthony, 1988), but the disadvantage of using pelage coloration is that differences in color are often subtle and subjective. Even after accounting for observer subjectivity (i.e., by using a standard color atlas and standard lighting), variation in pelage color can make distinction difficult. Notwithstanding, experienced researchers can use changes in color to supplement age predictions obtained by other means. Generally, the pelage of young bats is darker, less dense, and finer than in adults.

Existing Techniques for Age Estimation in Adults

Tooth Wear

In most bat species, deciduous teeth are replaced by a permanent set by the time juveniles are able to fly and feed independently. Once erupted, permanent teeth reach full size and then cease to grow. Mastication over the lifespan of an individual continually wears down tooth surfaces and thus erodes the enamel. Ultimately, cheek teeth become short as cusps are worn down, canines become short and dull, and the width of the tip increases. Because this process occurs from weaning until death, the degree of tooth wear can be used to place bats into relative age categories. Reference standards based on tooth wear have been developed for a few species including *Tadarida brasiliensis* (Davis et al., 1962), *Eptesicus fuscus* (Christian, 1956), *Myotis velifer* (Twente, 1955), *M. lucifugus* (Stegeman, 1956), and *Phyllostomus hastatus* (McCracken and Bradbury, 1981), *Artibeus jamaicensis* (Kunz et al., 1983), and *Cynopterus sphinx* (Storz et al., 2000). Using this method, these authors categorized bats into three to six age classes.

Scrutiny of the first developed standards established the limitations of their predictive power due to extensive variation of tooth wear patterns (Hall et al., 1957) and the generation of reference standards that lacked an adequate series of known-age specimens. Further attempts at improving the accuracy of estimates included using statistical methods to estimate maximum age ranges for tooth wear classes (Davis et al., 1962) and the development of quantitative indices that include wear patterns of more than one tooth (Christian, 1956; Sluiter, 1961; Baagøe, 1977). Neither of these approaches resulted in more precise predictions of age. Researchers still encountered significant overlap in tooth wear indices among bats of various age groups (as determined by other methods) and reference standards are primarily biased to younger age groups. It should also be noted that tagged bats known to be old often have teeth that are in very good condition (Hall et al., 1957).

Noticeable differences in tooth wear occur over long time periods that limit how narrow age categories based on this technique can be. Confounding results are the significant variation of tooth wear associated with differences in diet, use of teeth in intraspecific aggressive interactions, and latitudinal differences in annual activity periods. Moreover, obtaining accurate and repeatable tooth measurements on live animals can be cumbersome and difficult, especially under poor lighting conditions. Notwithstanding, the method has proven useful for a variety of behavioral and ecological studies where broad age categories suffice to obtain meaningful results. Critical for the appropriate use of this method is the development of reference standards based on a large sample of known-age individuals covering a broad age spectrum and that age estimates be made by investigators with extensive experience assessing tooth wear in the particular species in question.

Methods used to measure tooth wear (especially of canine teeth) range from measuring the height of the crown from the gum line or cingulum, measuring the cross-sectional diameter of the cusp with dial calipers (Kunz et al., 1983; Storz et al., 2000), or using clay or dental wax to make dental impressions and then measuring the height of the crown or width of the cusp (see Kunz et al., 1996). Otoscopes can also be used for observing dentition of live bats to assess and assign relative age indices based on degree of wear.

Incremental Dentin and Cementum Lines

Christian (1956) and Klevezal and Kleinenberg (1967) proposed the use of incremental lines or "annuli" in teeth to estimate the bats' ages. These incremental lines result from appositional growth of teeth, where new dentin and cementum are deposited on pre-existing tissue, and can be counted in histological sections of teeth. Klevezal and Kleinenberg found that the number of incremental lines counted on a sectioned tooth equaled the age of a bat in years. This method has been used satisfactorily in *Desmodus rotundus* (Linhart, 1973; Lord et al., 1976), *Nyctalus noctula*, *M. myotis* (Klevezal and Kleinenberg, 1967), and several other vespertilionid species (Baagøe, 1977; Schowalter et al., 1978; Funakoshi and Uchida, 1982). The robustness of the incremental lines varies among species, however, so that in some the lines may be difficult to count,

especially in small species. Phillips et al. (1982) examined incremental lines in two species of bats (*Myotis lucifugus* and *M. velifer*) from known-age specimens. They found that the number of incremental lines observed depended on the tooth that was extracted and on the section examined, and suggested that several factors, such as mechanical stress and dental drift, can affect the temporal patterns of appositional growth resulting in non-annual cycles of dentin and cementum deposition. Batulevicius et al. (2001) examined incremental lines in dentin and cementum in *Myotis daubentonii*, *Barbastella barbastella*, *M. brandtii*, *M. nattereri*, *Pipistrellus nathusii*, *Plecotus auritus*, *M. dasycneme*, and *Vespertilio murinus* and concluded that incremental lines in dentin were often too small to count accurately and were often not present in cementum. Of 26 individuals examined, three were of known age based on marking data, and number of incremental lines significantly underestimated their age. Examining canines from *Pteropus alecto* and *Pteropus poliocephalus*, Cool et al. (1994) found an average of 1.4 cemental layers forming every year in these species. Other researchers have found a lack of correlation between age and incremental lines in *Pipistrellus pipistrellus* and *N. noctula* (Baagøe, 1977).

Aside from variation in incremental lines and the questionable accuracy of this method, other disadvantages are that it requires tooth extraction, which can only be done on dead specimens, it is time consuming, and it requires specialized equipment. The problems associated with this method highlight the need to better understand how and when these incremental lines are deposited and use this information to develop more accurate reference standards that can be compared with other methods.

Size of Pulp Cavity

Posteruptive appositional growth of mammalian teeth occurs in such a way that new layers of dentin form in the pulp cavity. The addition of successive layers results in decreased size of the cavity over time, making the size of the pulp cavity a potential index for estimating age in bats. Baagøe (1977) developed this technique and tested it on a sample of four vespertilionid species. Extracted canines were suspended in glycerin, which makes them translucent enough to measure the width of the pulp cavity under a dissecting microscope using an ocular micrometer. While the ages estimated with this technique correlated with estimates based on tooth wear and condition of metacarpal and phalangeal epiphyses, the method has not been tested using known-age bats. Until it is tested, the accuracy and precision of the estimates remain questionable. One possible reason why this technique has not been further tested is that it requires the extraction of a canine (although Baagøe suggested that the pulp cavity can be visualized in live individuals of some small species) and the equipment needed limits its use in field situations. Additionally, deposition of dentin appears to decrease in adults (Baagøe, 1977), which may limit the use of this technique in older individuals.

Population-level Methods

Some studies, especially those investigating senescence and determinants of longevity, may be interested in average lifespan of a population, rather than specific ages of individuals. Life tables constructed with known-aged individuals or age cohorts can be used with demographic techniques to generate estimates of mean lifespan, life expectancy, survivorship rates, and age-specific mortality rates. The reader is directed to O'Donnell (this volume) for methods used to estimate these population parameters.

SENESCENCE AND LONGEVITY IN BATS

Senescence is the physical deterioration experienced by an organism as it gets older. At a population level, senescence is often manifested as an increase in age-specific mortality (actuarial senescence), which results as physical deterioration hampers an individual's ability to survive disease, predation, extreme weather, variable food availability, etc. Longevity is the total time from birth to death of an individual. Senescence and longevity are linked because decreasing the rate of senescence of an individual increases its longevity. Long lifespan of bats have been known for several decades (Bourliere, 1958; Tuttle and Stevenson, 1982), yet surprisingly few studies have focused on investigating this extraordinary longevity. The primary models of aging research have been lab mice and rats, fruit flies, and round worms, and only recently have researchers begun to recognize the value of exploring aging in a variety of organisms spanning a wide phylogenetic spectrum. Being among the longest-lived mammals, after adjusting for body size and metabolic rate, bats are a natural choice for investigations aimed at understanding mechanisms underlying long lifespan and reduced rates of senescence. Other advantages of using bats as model systems for aging studies are that many species can be successfully maintained in captivity (see Barnard, this edition); many are heterothermic, allowing researchers to investigate the role of metabolism on aging (see Willis and Cooper, this volume; Voigt and Cruz-Neto, this volume); and wild populations can be marked and monitored for long-term studies (see Kunz, Betke, and Hristov, and Vonhof, this volume).

Among the few studies that have explored bat longevity, most have focused on the question of why bats live so long. The exceptional longevity of bats is consistent with the evolutionary theory of aging, which attributes aging to the decreasing strength of natural selection with increasing age (Medawar, 1952; Williams, 1957; Charlesworth, 1980). This reduced selective pressure allows for the accumulation of late-acting deleterious alleles in the genome

that ultimately lead to senescence. Organisms that escape extrinsic mortality such as disease, predators, starvation, and accidents, evolve to live long because natural selection remains robust into later ages. Bats are successful at escaping extrinsic mortality owing to their ability to fly and use of protected roosts (Kunz, 1982; Kunz and Lumsden, 2003). In fact, there is a positive correlation between the longevity of bats and their use of caves, which afford bats protection from predators and extreme weather (Wilkinson and South, 2002). Hibernation also appears to extend longevity by allowing bats to escape severe weather conditions and food shortages, thereby delaying physiological deterioration. In fact, the average maximum lifespan of several hibernating bat species is six years longer than that of non-hibernating species (Wilkinson and South, 2002). Prolonged bat longevities are also consistent with the disposable soma theory of aging (Kirkwood, 1977, 1996). This theory postulates that longevity is the result of an inevitable tradeoff between investing limited resources and energy into somatic maintenance and reproduction. Longevity is lower in bat species with high reproductive rates (Rachmatulina, 1992; Wilkinson and South, 2002) and early sexual maturation (Rachmatulina, 1992). This tradeoff appears evident within a species as well. Ransome (1995) found that female horseshoe bats (*Rhinolophys ferrumequinum*) that delay breeding have higher survival rates than those that breed early in life.

Studies that have addressed the molecular or physiological mechanisms underlying bat longevity are limited. The first was a comparative study that addressed a hypothesized but intensely disputed (Cristofalo and Pignolo, 1995) correlation between the limited replicative lifespan of fibroblasts and the maximum lifespan of an organism. Using fibroblast cultures of eight different mammalian species, Röhme (1981) found a positive correlation between species' maximum longevity and the number of population doublings of cultured fibroblasts. It should be noted, however, that Röhme used only one individual of *Vespertilio murinus* for this study, so assessing intraspecific or interspecific variation was not possible. A recent reexamination of this correlation using skin fibroblast cultures from 11 mammalian species including *Eptesicus fuscus* found cellular replicative capacity to correlate primarily with species body size and not longevity (Lorenzini et al., 2005). While a correlation between bat longevity and cellular replicative lifespan is not supported, a study examining in vitro cellular resistance to lethal stresses presented evidence that fibroblasts from *M. lucifugus* are particularly resistant to death induced by exposure to cadmium, hydrogen peroxide, and methanesulfonate. These bat cells were also resistant to effects of the mitochondrial inhibitor retonone (Harper et al., 2007).

Baudry et al. (1986) tested the hypothesis that low brain calpain activity is related to increased longevity. The calpains are a family of calcium-dependent cysteine proteases involved in a variety of cellular functions, and inappropriate activity of calpain is implicated in several age-related pathologies (Bahr et al., 1991; Vanderklish and Bahr, 2000). Calpain activity from brain tissues of two bat species, *Antrozous pallidus* and *Tadarida brasiliensis*, was significantly lower than in brain tissues from mice. Investigation of proteins such as calpains may provide insight into the mechanisms leading to age-related deterioration of the brain; however, the exact link between calpain activity and longevity (if a direct link exists) remains to be elucidated.

A couple of studies have tested a hypothesis of aging that has received much attention. The free radical theory of aging ascribes aging to the accumulation of unrepaired oxidative damage to cellular components caused by reactive oxygen species (ROS), which are generated mostly during cellular respiration (Harman, 1956; Sohal, 1986). Although still debated, analyses of oxidative stress suggest that ROS production is a better predictor of longevity than antioxidant activity (Barja, 2002). Brunet-Rossinni (2004) conducted a study that compared the production of hydrogen peroxide in mitochondria (a measure of ROS production) of tissues from the little brown myotis (*M. lucifugus*), short-tailed shrew (*Blarina brevicauda*), and white-footed mouse (*Peromyscus leucopus*). The mitochondria from this bat produced significantly lower levels of hydrogen peroxide per unit of oxygen consumed than mitochondria from the shrew and mouse, while activity of superoxide dismutase, a critical antioxidant, was similar in tissue homogenates from all three species. Lambert et al. (2007) compared hydrogen peroxide production in heart tissue of 12 mammalian and avian species, including *M. lucifugus* and *T. brasiliensis*. Their study confirmed an inverse correlation between mitochondrial free radical production and longevity even after removing possible confounding effects of body mass and phylogeny.

Mitochondrial DNA (mtDNA) is believed to be particularly susceptible to oxidative damage because it is near the source of ROS production and lacks the protection, such as histone proteins, and some of the repair systems that are present in the nucleus (Wanagat et al., 2001). Estimates for the frequency of oxidative damage to mtDNA range from 10^4 per cell per day in humans to 10^3 per cell per day in mice and rats (Ames, 1989; Fraga et al., 1990). A number of studies that have involved multiple species have found that deletions in mtDNA sequence accumulate with age (Melov et al., 1995; Schwarze et al., 1995; Liu et al., 1998; Esposito et al., 1999; Wanagat et al., 2001). Because the mammalian mitochondrial genome encodes at least 13 polypeptides that participate in the electron transport chain, damage to any of these genes, or to regions that regulate replication or transcription, should adversely affect oxidative phosphorylation and, therefore, metabolic rates. Not surprisingly, considerable evidence indicates that mitochondrial function declines with age (Wallace, 1999; Wanagat et al.,

2001) in most mammals, although several studies provide comparative genomic data suggesting that some long-lived bats may have a mechanism for repairing part of the mitochondrial control region, which is critical for successful replication and transcription.

In a survey of variation in mitochondrial sequences and length among bats (Wilkinson et al., 1997), every species of vespertilionid bat, many of which have recorded longevities in excess of 20 years (Wilkinson and South 2002; Gaisler et al., 2003), but no species from any other family, possessed 2–9 tandemly arrayed copies of a 78–85 bp portion of the mtDNA control region. This region contains noncoding sequences involved in controlling replication (Wilkinson et al., 1997). Interestingly, *Myotis lucifugus*, which has been recorded surviving for 34 years, had one of the highest average copy numbers of any species examined. One possible advantage to carrying duplications of this region is that they potentially could provide a method for repairing damage to protein binding sites that are important for replication. Repair could occur by the same mechanism that creates variation in length of these arrays. Each 80 bp repeat contains a complimentary sequence that can fold upon itself to create a stable secondary structure during replication, which can then either undergo a deletion or duplication event (Buroker et al., 1990; Wilkinson and Chapman, 1991). A deletion followed by a duplication event could, therefore, replace point mutations within a repeat. Several lines of evidence—including high levels of heteroplasmy (the occurrence of multiple types of mitochondrial genomes within an organism) and sequence homogeneity among internal repeats—indicate that duplication and deletion events commonly occur in vespertilionid bats (Wilkinson and Chapman, 1991; Petri et al., 1996; Wilkinson et al., 1997). Experimental evidence indicating that this process influences the accumulation of oxidative damage in proteins or mtDNA sequence remains to be obtained. This process also clearly cannot account for the extreme longevity observed in a few bats from other families, such as *Rhinolophus ferrumequinum*.

FUTURE LINES OF RESEARCH

In our view, there are two major gaps in our knowledge of aging and senescence in bats. First, a consistent and well-tested method for obtaining accurate age estimates of adult bats is necessary. The currently available methods are limited to placing individual bats into age categories that can encompass several chronological ages. The variable ages of individuals in a category can introduce variation in the trait being studied potentially resulting in misleading conclusions. Perhaps accumulated oxidative DNA damage or lipid and protein peroxidation damage correlates linearly with age, as suggested by the free radical theory of aging. If so, it may be possible to use small tissue biopsies and measure the accumulated products of this damage (e.g., lipofuscin) as estimators of age. To date, this area of research remains unexplored in regards to bat species.

Second, the few studies to date on the aging process in bats have focused exclusively on determinants of longevity; that is, why and how bats live so long (Brunet-Rossinni and Austad, 2004). Aside from tooth wear, we know nothing about senescence—physical deterioration with age—in bats. For example, what do old bats die from? Many strains of laboratory mice selected for longevity die of cancer. Do bats get cancer? The heart of heterothermic bats is exceptional in its ability to tolerate a broad range of beating rates (Pauziene et al., 2000) and withstand periods of hypoxia and hypercarbia during arousal from hibernation (Kallen, 1977). Does this cardiac functional flexibility extend into old age? In humans, a high fat diet is correlated with cardiac disease and atherosclerosis. The diet of the Brazilian free-tailed bat may be comprised of 60% fat (Kunz et al., 1995), but Widmaier et al. (1996) found no evidence of plaque formation in the coronary arteries or aortas of this species. How are these animals protected? What mechanism allows them to clear cholesterol and triglycerides from their bloodstream? Hyperglycemia and hyperinsulinemia are often associated with age-related diseases (Masoro, 1996). Frugivorous and nectivorous bats ingest and quickly assimilate large amounts of sugar every night and tolerate a broad range of blood sugar levels (Keegan, 1977). Do bats retain this metabolic plasticity into old age? Do they develop diabetes?

Do bats experience sensory loss, decreased reproductive success, and compromised immune function with age as do many other mammals? Auditory acuity is critical for Microchiroptera, as they rely on echolocation for foraging and navigation. If some individuals survive over 30 years, then auditory sensitivity must be preserved into old age, which is remarkable considering the high frequency and intensity of echolocation calls (Neuweiler, 2000). Kirkegaard and Jørgensen (2000) presented preliminary evidence of turnover in hair cells in the inner ear of *M. daubentonii*. How extensive is this turnover? Does it last into old age? Some mammals, especially primates, show evidence of reproductive senescence, a decrease in reproductive output with age, and immunosenescence, a decrease in immune response and effectiveness with age (Brunet-Rossinni and Austad, 2005). After controlling for year-to-year variation in body condition, does the rate of reproduction of a female bat change with age? How does the primary immune response of an older bat to an unknown immune challenge compare to that mounted by a young bat? Do older bats mount a more successful secondary immune response?

The above is a small sample of questions that if answered may contribute to our understanding of universal mechanisms underlying senescence and longevity in bats,

and will significantly improve our knowledge of their biology. In our judgment, the primary reason for our limited understanding of senescence in bats is the need for accurate knowledge of chronological age. At present, the only method available to obtain this information is to permanently mark individuals at birth and then to monitor these individuals over several years. While there are few long-term monitoring studies of bat populations, new information is needed to advance our knowledge of senescence and age estimation methods. We hope that the inclusion of these questions will promote an interest in pursuing these lines of research and that this, in return, will lead to the development of accurate and reliable methods of age estimation in adult bats.

ACKNOWLEDGMENTS

Authors are very grateful to T. H. Kunz and two anonymous reviewers for their helpful comments on drafts of the manuscript.

LITERATURE CITED

Ames, B. N. 1989. Endogenous DNA damage as related to cancer and aging. Mutation Research 214: 41–46.

Anthony, E. L. P. 1988. Age determination in bats. Pp. 47–58, In: Ecological and Behavioral Methods for the Study of Bats (T. H. Kunz, ed.). Smithsonian Institution Press, Washington, DC.

Austad, S. N., and K. E. Fischer. 1991. Mammalian ageing, metabolism and ecology: evidence from the bats and marsupials. Journal of Gerontology 46: B47–B53.

Baagøe, H. J. 1977. Age determination in bats (Chiroptera). Videnskabelige Meddelelser fra Danska Naturhistorisk Forening 140: 53–92.

Bahr, B. A., P. W. Vanderklish, L. T. Ha, M. Tin, M. Kessler, and G. Lynch. 1991. Spectrin breakdown products increase with age in telencephalon of mouse brain. Neuroscience Letters 131: 237–240.

Baptista, T. L., C. S. Richardson, and T. H. Kunz. 2000. Postnatal growth and age estimation in free-ranging bats: a comparison of longitudinal and cross-sectional sampling methods. Journal of Mammalogy 81: 709–718.

Barja, G. 2002. Rate of generation of oxidative stress-related damage and animal longevity. Free Radical Biology and Medicine 33: 1167–1172.

Batulevicius, D., N. Pauziene, and D. H. Pauza. 2001. Dental incremental lines in some small species of the European vespertilionid bats. Acta Theriologica 46: 33–42.

Baudry, M., R. DuBrin, L. Beasley, M. Leon, and G. Lynch. 1986. Low levels of calpain activity in Chiroptera brain: implications for mechanisms of aging. Neurobiology of Aging 7: 255–258.

Bourliere, M. D. 1958. The comparative biology of aging. Journal of Gerontology 13: 16–24.

Brunet-Rossinni, A. K. 2004. Reduced free-radical production and extreme longevity in the little brown bat (*Myotis lucifugus*) versus two non-flying mammals. Mechanisms of Aging and Development 125: 11–20.

Brunet-Rossinni, A. K., and S. N. Austad. 2004. Ageing studies on bats: a review. Biogerontology 5: 211–222.

———. 2005. Senescence in natural populations of mammals and birds. Pp. 243–265, In: Handbook of the Biology of Aging, 6th ed. (E. J. Masoro and S. N. Austad, eds.). Elsevier Academic Press, San Diego.

Burnett, C. D., and T. H. Kunz. 1982. Growth rates and age estimation in *Eptesicus fuscus* and comparison with *Myotis lucifugus*. Journal of Mammalogy 63: 33–41.

Buroker, N. E., J. R. Brown, T. A. Gilbert, P. J. O'Hara, A. T. Beckenbach, W. K. Thomas, and M. J. Smith. 1990. Length heteroplasmy of sturgeon mitochondrial DNA: an illegitimate elongation model. Genetics 124: 157–163.

Charlesworth, B. 1980. Evolution in Age-Structured Populations. Cambridge University Press, Cambridge, United Kingdom.

Cheng, H. C., and L. L. Lee. 2002. Postnatal growth, age estimation, and sexual maturity in the Formosan leaf-nosed bat (*Hipposideros terasensis*). Journal of Mammalogy 83: 783–793.

Christian, J. J. 1956. The natural history of a summer aggregation of the big brown bat, *Eptesicus fuscus fuscus*. American Midland Naturalist 55: 66–95.

Christofalo, V. J., and R. J. Pignolo. 1995. Cell culture as a model. Pp. 53–82, In: Handbook of Physiology Sect 11: Aging (E. J. Masoro, ed.). American Physiological Society, New York.

Cool, S. M., M. B. Bennett, and K. Romaniuk. 1994. Age estimation of pteropodid bats (Megachiroptera) from hard tissue parameters. Wildlife Research 21: 353–364.

Davis, R. B., C. F. Herreid II, and H. L. Short. 1962. Mexican free-tailed bats in Texas. Ecological Monographs 32: 311–346.

De Paz, O. 1986. Age estimation and postnatal growth of the greater mouse bat in Guadalajara, Spain. Mammalia 50: 243–251.

Elangovan, V., H. Raghuram, E. Yuvana Satya Priya, and G. Marimuthu. 2002. Postnatal growth, age estimation and development of foraging behavior in the fulvous fruit bat, *Rousettus leschenaulti*. Journal of Biosciences 27: 695–702.

Esposito, L. A., S. Melov, A. Panov, B. A. Cottrell, and D. C. Wallace. 1999. Mitochondrial disease in mouse results in increased oxidative stress. Proceedings of the National Academy of Sciences of the United States of America 96: 4820–4825.

Fraga, C. G., M. K. Shigenaga, J. W. Park, P. Degan, and B. N. Ames. 1990. Oxidative damage to DNA during aging - 8-Hydroxy-2'- deoxyguanosine in rat organ DNA and urine. Proceedings of the National Academy of Sciences of the United States of America 87: 4533–4537.

Funakoshi, K., and T. A. Uchida. 1982. Age composition of summer colonies in the Japanese house-dwelling bat, *Pipistrellus abramus*. Journal of the Faculty of Agriculture, Kyushu University 27: 55–64.

Gaisler, J., V. Hanák, V. Hanzal, and V. Jarsky. 2003. Results of bat banding in the Czech and Slovak republics 1948–2000. (In Czech, English summary.) Vespertilio 7: 3–63.

Hall, J. S., R. J. Cloutier, and D. R. Griffin. 1957. Longevity records and notes on tooth wear of bats. Journal of Mammalogy 38: 407–409.

Harman, D. 1956. Aging: a theory based on free radical radiation chemistry. Journal of Gerontology 11: 298–300.

Harper, J. M., A. B. Salmon, S. F. Leiser, A. T. Galecki, and R. A. Miller. 2007. Skin-derived fibroblasts from long-lived

—-1
—0
—+1

species are resistant to some, but not all, lethal stresses and to the mitochondrial inhibitor rotenone. Aging Cell 6: 1–13.

Hoying, K. M., and T. H. Kunz. 1998. Variation in size at birth and post-natal growth in the insectivorous bat *Pipistrellus subflavus* (Chiroptera: Vespertilionidae). Journal of Zoology (London) 245: 15–27.

Isaac, S. S., and G. Marimuthu. 1996. Postnatal growth and age estimation in the Indian pygmy bat, *Pipistrellus mimus*. Journal of Mammalogy 77: 199–204.

Kallen, F. C. 1977. The cardiovascular system of bats: structure and function. Pp. 289–483, In: Biology of Bats (W. A. Wimsatt, ed.). Academic Press, New York.

Keegan, D. J. 1977. Aspects of the assimilation of sugars by *Rousettus aegyptiacus*. Comparative Biochemistry and Physiology A 58: 349–352.

Kirkegaard, M., and J. M. Jørgensen. 2000. Continuous hair cell turnover in the inner ear vestibular organs of a mammal, the Daubenton's bat (*Myotis daubentonii*). Naturwissenschanften 87: 83–86.

Kirkwood, T. B. L. 1977. Evolution of ageing. Nature 270: 301–304.

———. 1996. Human senescence. BioEssays 18: 1009–1016.

Klevezal, G. A., and S. E. Kleinenberg. 1967. Age determination of mammals from annual layers in teeth and bones. (Translated from Russian.) Israel Program for Scientific Translations, Jerusalem (1969), TT 69–55033.

Koehler, S. E., and R. M. R. Barclay. 2000. Post-natal growth and breeding biology of the hoary bat (*Lasiurus cinereus*). Journal of Mammalogy 81: 234–244.

Kunz, T. H. 1982. Roosting ecology of bats. Pp. 1–55, In: Ecology of Bats (T. H. Kunz, ed.). Plenum Press, New York.

Kunz, T. H., and E. L. P. Anthony. 1982. Age estimation and post-natal growth in the bat *Myotis lucifugus*. Journal of Mammalogy 63: 23–32.

Kunz, T. H., and S. K. Robson. 1995. Postnatal growth and development in the Mexican free-tailed bat (*Tadarida brasiliensis mexicana*): birth size, growth rates and age estimation. Journal of Mammalogy 76: 769–783.

Kunz, T. H., and A. A. Stern. 1995. Maternal investment and post-natal growth in bats. Symposia of the Zoological Society of London, No. 67: 123–138.

Kunz, T. H., and W. R. Hood. 2000. Parental effort and postnatal growth in the Chiroptera. Pp. 415–468, In: Reproductive Biology of Bats (E. G. Crichton and P. H. Krutzsch, eds.). Academic Press, San Diego.

Kunz, T. H., and L. F. Lumsden. 2003. Ecology of cavity and foliage roosting bats. Pp. 3–89, In: Bat Ecology (T. H. Kunz and M. B. Fenton, eds.). University of Chicago Press, Chicago.

Kunz, T. H., P. V. August, and C. D. Burnett. 1983. Harem social organization in cave roosting *Artibeus jamaicensis* (Chiroptera: Phyllostomidae). Biotropica 15: 133–138.

Kunz, T. H., J. O. Whitaker, Jr., and M. D. Wadanoli. 1995. Dietary energetics of the Mexican free-tailed bat (*Tadarida brasiliensis*) during pregnancy and lactation. Oecologia 101: 107–115.

Kunz, T. H., C. D. Wemmer, and V. Hayssen. 1996. Sex, age, and reproductive condition of mammals. Pp. 279–290, In: Measuring and Monitoring Biological Diversity: Standard Methods for Mammals (D. E. Wilson, F. R. Cole, J. D. Nichols, R. Rudran, and M. S. Foster, eds.). Smithsonian Institution Press, Washington, DC.

Lambert, A. J., H. M. Boysen, J. A. Buckingham, T. Yang, A. Podlutsky, S. N. Austad, T. H. Kunz, R. Buffenstein, and M. D. Brand. 2007. Low rates of hydrogen peroxide production by isolated heart mitochondria associate with long maximum lifespan in vertebrate homeotherms. Aging Cell 6: 607–618.

Linhart, S. B. 1973. Age determination and occurrence of incremental growth lines in the dental cementum of the common vampire bat (*Desmodus rotundus*). Journal of Mammalogy 54: 493–496.

Liu, V. W. S., C. Zhang, and P. Nagley. 1998. Mutations in mitochondrial DNA accumulate differentially in three different human tissues during ageing. Nucleic Acids Research 26: 1268–1275.

Lord, R. D., F. Muradall, and L. Lazaro. 1976. Age composition of vampire bats (*Desmodus rotundus*) in northern Argentina and southern Brazil. Journal of Mammalogy 57: 573–575.

Lorenzini, A., M. Tresini, S. N. Austad, and V. J. Cristofalo. 2005. Cellular replicative capacity correlates primarily with species body mass not longevity. Mechanisms of Ageing and Development 126: 1130–1133.

Masoro, E. J. 1996. Possible mechanisms underlying the antiaging actions of caloric restriction. Toxicology and Pathology 24: 738–741.

McCracken, G. F., and J. W. Bradbury. 1981. Social organization and kinship in the polygynous bat *Phyllostomus hastatus*. Behavioral Ecology and Sociobiology 8: 11–34.

Medawar, P. B. 1952. An Unsolved Problem in Biology. H. K. Lewis, London.

Melov, S., J. M. Shoffner, A. Kaufman, and D. C. Wallace. 1995. Marked increase in the number and variety of mitochondrial DNA rearrangements in aging human skeletal muscle. Nucleic Acids Research 23: 4122–4126.

Neuweiler, G. 2000. The Biology of Bats. Oxford University Press, New York.

Parry-Jones, K. 2002. Age estimation and confounding variations in growth rates of the grey headed flying fox, *Pteropus poliocephalus*. Bat Research News 43: 20.

Pauziene, N., D. H. Pauza, and R. Stropus. 2000. Morphological study of the heart innervation of bats *Myotis daubentonii* and *Eptesicus serotinus* (Microchiroptera: Vespertilionidae) during hibernation. European Journal of Morphology 38: 195–205.

Petri, B., A. Vonhaeseler, and S. Paabo. 1996. Extreme sequence heteroplasmy in bat mitochondrial DNA. Biological Chemistry 377: 661–667.

Phillips, C. J., B. Steinberg, and T. H. Kunz. 1982. Dentin, cementum, and age determination in bats: a critical evaluation. Journal of Mammalogy 63: 197–207.

Rachmatulina, I. K. 1992. Major demographic characteristics of populations of certain bats from Azerbaijan. Pp. 127–141, In: Prague Studies in Mammalogy (J. Horacek and V. Vohralik, eds.). Charles University Press, Prague, Czech Republic.

Rajan, K. E., and G. Marimuthu. 1999. Postnatal growth and age estimation in the Indian false vampire bat (*Megaderma lyra*). Journal of Zoology (London) 248: 529–534.

Ransome, R. D. 1995. Earlier breeding shortens life in female greater horseshoe bats. Philosophical Transactions of the Royal Society of London, Series B 350: 153–161.

Richardson, P. 1990. Assessing age and breeding status of Daubenton's bat, *Myotis daubentoni*. Bat Research News 31: 47–48.

Röhme, D. 1981. Evidence for a relationship between longevity of mammalian species and lifespans of normal fibroblasts *in vitro* and erythrocytes *in vivo*. Proceedings of the National Academy of Sciences of the United States of America 78: 5009–5013.

Rybár, P. 1969. Ossification of bones as age criterion in bats (Chiroptera). (In Czech, English summary.) Pracé a studie Prirodni, Pardubice 1: 115–136.

———. 1971. On the problems of practical use of the ossification of bones as age criterion in the bats (Microchiroptera). (In Czech, English summary.) Práce a studie-Prirodni, Pardubice 3: 97–121.

Schowalter, D. B., L. D. Harder, and B. H. Treichel. 1978. Age composition of some vespertilionid bats as determined by dental annuli. Canadian Journal of Zoology 56: 355–358.

Schwarze, S. R., C. M. Lee, S. S. Chung, E. B. Roecker, R. Weindruch, and J. M. Aiken. 1995. High levels of mitochondrial DNA deletions in skeletal muscle of old rhesus monkeys. Mechanisms of Ageing and Development 83: 91–101.

Sharifi, M., B. Kiabi, and K. Faizolahi. 2002. Postnatal growth in the lesser mouse-eared bat *Myotis blythii* in a maternity roost in western Iran. Bat Research News 43: 109.

Sluiter, J. W. 1961. Abrasion of teeth in connection with age in the bat *Myotis myotis*. Proceedings of the Koninklijke Nederlandse Akademie van Wetenschappen C 64: 424–434.

Sohal, R. S. 1986. The rate of living theory: a contemporary interpretation. Pp. 23–44, In: Insect Aging: Strategies and Mechanisms (K. G. Collatz and R. S. Sohal, eds.). Springer-Verlag, Berlin, Germany.

Stegeman, L. C. 1956. Tooth development and wear in *Myotis*. Journal of Mammalogy 37: 58–63.

Stern, A. A., and T. H. Kunz. 1998. Intraspecific variation in postnatal growth in the greater spear-nosed bat. Journal of Mammalogy 79: 755–763.

Storz, J. F., H. R. Bhat, and T. H. Kunz. 2000. Social structure of a polygynous tent-making bat, *Cynopterus sphinx* (Megachiroptera). Journal of Zoology (London) 250: 151–165.

Tuttle, M. D., and D. Stevenson. 1982. Growth and survival of bats. Pp. 105–150, In: Ecology of Bats (T. H. Kunz, ed.). Plenum Press, New York.

Twente, J. W. 1955. Aspects of a population study of cavern-dwelling bats. Journal of Mammalogy 36: 379–390.

Vanderklish, P. W., and B. A. Bahr. 2000. The pathogenic activation of calpain: a marker and mediator of cellular toxicity and disease states. International Journal of Experimental Pathology 81: 323–339.

Wallace, D. C. 1999. Mitochondrial diseases in man and mouse. Science 283: 1482–1488.

Wanagat, J., M. Lopez, and J. M. Aiken. 2001. Alterations of the mitochondrial genome. Pp. 114–139, In: Handbook of the Biology of Aging (E. J. Masoro and S. N. Austad, eds.). Academic Press, New York.

Widmaier, E. P., E. R. Gornstein, J. L. Hennessey, J. M. Bloss, J. A. Greenberg, and T. H. Kunz. 1996. High plasma cholesterol, but low triglycerides and plaque-free arteries, in Mexican free-tailed bats. American Journal of Physiology 271: R1101–R1106.

Wilkinson, G. S., and A. S. Chapman. 1991. Length and sequence variation in evening bat d-loop mtDNA. Genetics 128: 607–617.

Wilkinson, G. S., and J. M. South. 2002. Life history, ecology and longevity in bats. Aging Cell 1: 124–131.

Wilkinson, G. S., F. Mayer, G. Kerth, and B. Petri. 1997. Evolution of repeated sequence arrays in the D-loop region of bat mtDNA. Genetics 146: 1035–1048.

Williams, G. C. 1957. Pleiotropy, natural selection and the evolution of senescence. Evolution 11: 398–411.