

# The evolution of aging phenotypes in snakes: a review and synthesis with new data

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**Abstract** Reptiles are underutilized vertebrate models in the study of the evolution and persistence of senescence. Their unique physiology, indeterminate growth, and increasing fecundity across the adult female lifespan motivate the study of how physiology at the mechanistic level, life history at the organismal level, and natural selection at the evolutionary timescale define lifespan in this diverse taxonomic group. Reviewed here are, first, comparative results of cellular metabolic studies conducted across a range of colubrid snake species with variable lifespan. New results on the efficiency of DNA repair in these species are synthesized with the cellular studies. Second, detailed studies of the ecology, life history, and cellular physiology are reviewed for one colubrid species with either short or long lifespan (*Thamnophis elegans*). New results on the rate of telomere shortening with age in this species are synthesized with previous research. The comparative and intraspecific studies both yield results that species with longer lifespans have underlying cellular physiologies support the free-radical/repair mechanistic hypothesis for aging. As well, both underscore the importance of mortality environment for the evolution of aging rate.

**Keywords** Aging · Comet assay · Comparative biology · DNA damage and repair · Free radical theory · Evolution · Mitochondrial efficiency · Oxidative damage · Respiratory coefficient ratio · Senescence · Telomeres · Reptiles · Snakes

## Abbreviations

SCGE Single cell gel electrophoresis  
TRF Telomere restriction fragment

## Introduction

Evolutionary theory demonstrates that the minimum requirement for senescence to evolve in a population is a cohort survival pattern of fewer individuals surviving to each new age class (Charlesworth 1994). At the population level, this leads to fewer old than young individuals. Given this survival pattern, mutations that enhance late-life fitness at the cost of early-life fitness will have a smaller net impact on population viability than mutations that enhance early-life fitness at the cost of late-life fitness. The declining number of individuals in a cohort over time may be due to external sources of mortality acting without respect to age, such as predation, starvation or accidents, or may be due to increasing intrinsic mortality risk with age. This survival pattern, and the resulting changes in selection differentials with age across the lifespan, were shown by Hamilton (1966) to be the raw ingredients for

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senescence to evolve. The evolution of the rate of senescence, in turn, is controlled by the trajectory of age specific mutation/selection balances. The shape of these trajectories hinges on the mortality environment that a species, or different populations within a species, experiences over generations. Senescence, and the rate of this intrinsic decline and mortality, is an evolvable intrinsic phenotype dependent on extrinsic source and force of mortality (reviewed in Promislow and Bronikowski 2006).

More recent theoretical considerations of how senescence evolves have allowed for the possibility that enhanced fecundity at late ages can combat declining selection with age. This can change the strength of selection enough to promote prolonged somatic maintenance and reproduction, and allow the evolution of negligible senescence (Vaupel et al. 2004; Baudisch 2005). Recent empirical work on organisms with indeterminate growth and fecundity suggests that increasing fecundity with advancing age may override decreasing survival with advancing age in these species, such that they may escape senescence altogether (Reznick et al. 2004; Sparkman et al. 2007).

Indeterminately growing reptiles in general, and snakes in particular, are prime candidates for aging research (e.g., Kardong 1996; Congdon et al. 2001, 2003; Miller 2001). Snakes are underutilized models for studying the evolution of aging, in spite of the fact that a variety of remarkable characteristics recommend them as vertebrate animal models (Olsson and Shine 1996; Ujvari and Madsen 2006). In addition to indeterminate growth, they have the ability to shut down their metabolism for long periods of time, which likely releases them from costs of catch-up growth like those documented in birds (reviewed in Metcalfe and Monaghan 2003). Furthermore, snakes are plastic in terms of their age of sexual maturation. Many snake species exhibit increasing fecundity with advancing age, perhaps as a result of learning and experience, but more likely through a direct physiological effect of increased reproductive output with increasing body size (reviewed in Sparkman et al. 2007). For all these reasons, and because of reported extreme longevities, reptiles are expected to yield insights into the evolution of senescence from the perspective of studying a taxonomic group that may not senesce (e.g., Congdon et al. 2003; Lance 2003; Robert et al. 2007).

In addition to being neglected in ultimate or evolutionary aging studies, reptiles are an underutilized

model for studying the proximate physiological and cellular mechanisms of aging. Although their longevities often preclude following cohorts until death, the repeated measurement of various dependent variables associated with aging throughout the lifetime of individuals suggests that reptiles have unusual means of coping with normal energetic stresses (e.g., thermal variation) as well as stresses thought to induce the flight or fight stress response (e.g., predation). Snakes, in particular, have been a research window into the evolution of various physiological and morphological adaptations potentially relevant to aging phenomena, including starvation resistance (e.g., shutting down the digestive tract: Secor et al. 1994; Secor and Diamond 1998), cold and heat tolerance (e.g., non-emergence from over-wintering hibernacula: Bronikowski and Arnold 1999), and predation avoidance (e.g., venom and mimicry, Brodie 1993; Kardong 1996; Fry and Wüster 2004). They also exhibit a large degree of variation in lifespan, both within and among closely related species.

While reptile species differ in documented maximum lifespans, we have no idea whether this is due to the process of senescence—that is, the breakdown of physiological, biochemical, morphological, and/or performance characteristics with age. Research in my laboratory on aging in reptiles addresses two questions. First, if senescence occurs in reptiles, how do indeterminate growth and fecundity factor into predictions regarding expected rates of aging? Second, if reptiles do not senesce, what cellular phenomena support this apparent negligible senescence? Here, I first review our findings from comparative studies of colubrid snake species with additional new data on inducible DNA damage. Second, I review detailed physiological measurements within one of these species with remarkable lifespan variation and present new data on rate of telomere shortening with age. These inter- and intra-specific studies of a suite of dependent variables illustrate the importance of measuring indices of aging along multiple axes and at multiple levels of analysis: including the cellular, bioenergetic, and evolutionary.

### **Comparative analyses of lifespan correlates in colubrid snakes**

The snake family Colubridae contains over 2,000 species. Often referred to as “common snakes”, this

family consists of non-venomous, widely distributed snake genera that range in lifespan from 2 years (with likely only one reproductive event) to over 50 years. We have previously published studies on six colubrid species of snakes, sampled across the phylogenetic range of this family, ranging in lifespan from 7 to 30 years (Robert et al. 2007). The goal of the study was to test the free radical/repair theory of aging. This theory posits that aging, and ultimately death, results from the accumulation of damaged biomolecules with age due to a declining ability to repair damage with age (reviewed in Beckman and Ames 1998; Finkel and Holbrook 2000). The damaging forces can be such stressors as free radicals, UV radiation, heat, starvation, etc. The predecessor of the free radical theory, the rate of living hypothesis, tied lifespan and aging rate to metabolic rate (Pearl 1928). However, experimentation ultimately showed that it was not metabolic rate *per se* that drove oxidative stress, but the degree to which metabolism and other cellular processes that produced free radicals were offset by the cellular machinery for destroying these oxidants.

We undertook these comparative studies of snakes to test for positive relationships among metabolic rate, cellular respiration, and oxidative damage potential in an organism that can drop its metabolic rate both in the absence of food and at lower than ideal temperatures. We hypothesized that long-lived species of snakes would have lower mass-independent oxygen consumption, higher mitochondrial efficiency (respiratory coefficient ratios), and less leakage of superoxide anions—and therefore less production of hydrogen peroxide—during resting metabolism. In addition, we measured slither speed, which has been shown to predict juvenile survival in the wild, and which we hypothesized would be faster in long-lived species relative to short-lived species that utilize the same form of locomotion when foraging (Robert et al. 2007). Our findings supported the free radical hypothesis, in that longer-lived species of colubrid snakes did indeed have lower mitochondrial free radical production. Neither whole-organism nor mitochondrial oxygen consumption differed among species. Longer-lived species also exhibited faster slither performance. Since the time of that publication, we have had the opportunity to measure two other potentially important dependent variables, DNA damage and repair efficiency in erythrocytes from the same cohort of juveniles used in the initial study.

## DNA damage and repair

### DNA damage and repair: methods

DNA damage and repair were assessed using the comet assay (Singh et al. 1998; Tice 1995). Also called single cell gel electrophoresis (SCGE), the comet assay is a sensitive and rapid technique for quantifying and analyzing DNA damage in individual cells. Briefly, intact cells are embedded in a thin agarose gel on a microscope slide. All cellular proteins are then removed from the cells by lysing. The DNA is allowed to unwind under alkaline/neutral conditions. Following the unwinding, the DNA is subjected to electrophoresis, allowing the broken DNA fragments or damaged DNA to migrate away from the nucleus, forming a comet-like pattern. After staining with a DNA-specific fluorescent dye, the gel is quantified for amount of fluorescence in head and tail, and various aspects of the head and tail of the “comet.” The extent to which DNA is liberated from the head of the comet is directly proportional to the amount of DNA damage.

Comet slides (Trevigen, Gaithersburg, MD) were coated with 100  $\mu$ l 1% normal-melting-point agarose and dried overnight at room temperature. Pre-coated slides were warmed to 37°C, layered with 100  $\mu$ l 1.0% low-melting-point agarose and placed at 4°C for 10 min. Blood samples were mixed with agarose by first diluting whole blood samples pooled over three individuals per species. The species were the same as in Robert et al. 2007 (common names: corn snake, diadem snake, house snake, garter snake, trinket snake, king snake; see Results). The initial whole blood dilution was 10  $\mu$ l/990  $\mu$ l PBS. This diluted sample was then mixed as follows: 500  $\mu$ l of each sample with 500  $\mu$ l 1.5% low-melting-point agarose. Each sample was divided into three 150- $\mu$ l aliquots, immediately transferred to pre-warmed slides and gently covered with a cover slip to ensure an even distribution of cells in the agarose layer. The slides were placed at 4°C for layers to harden and layered with 100  $\mu$ l 1% low-melting-point agarose as the last layer. After 10 min at 4°C, the cover slips were carefully removed and the slides subjected to one of three treatments. The first of three sets of slides from each species, the “A” treatment, were lysed and electrophoresed without further manipulation to provide a measure of baseline DNA damage in eryth-

rocytes. The second of three sets of slides, the “B” treatment slides, were subjected to 312-nm UV light for 5 min prior to lysing and electrophoresis. The B slides provided a measure of inducible DNA damage. The third set of slides, “C” treatment, were exposed to UV light as in the B treatment and then allowed 10 min at room temperature prior to lysing for DNA repair to occur. To lyse the cells, all slides were placed in cold lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Trizma base) for at least 1 h at 4 °C. Afterward slides were washed 5 min×3 times in neutralization buffer (0.4 M Tris free base). Electrophoresis was performed in buffer (300 mM NaOH, 1 mM EDTA) at 25 V and 300 mA for 40 min at 4° C. After electrophoresis, the slides were washed in neutralization buffer again and the DNA fixed by incubation in 100% ethanol for 10 min. After air-drying, the slides were stained with SYBR Green for image analysis.

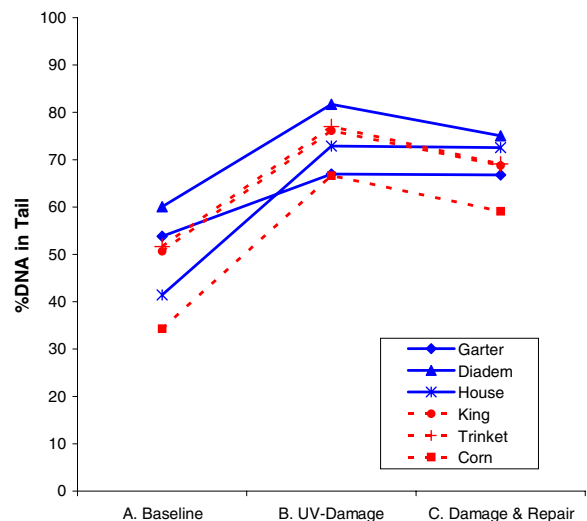
Stained lysed cells resemble comets when viewed under a fluorescence microscope. Fluorescent images were evaluated by image analysis software to calculate comet dependent variables (comet tail length, tail width, % DNA in tail vs head, tail Olive moment) for approximately 40 comets per treatment per species (678 comets in total due to inability to analyze a few comets per several species). The individual comet dependent variables were all highly correlated; results for % DNA in the comet tail are presented in Table 1.

#### DNA damage and repair: results

Analysis of variance of erythrocyte DNA damage revealed a significant species-by-treatment interaction for % DNA in the comet tail (Table 1). This can be interpreted as evidence that different species of snakes are responding differently to UV-irradiation and to DNA-repair opportunity. Overall, 5 min of UV irradiation was sufficient time to induce significant DNA damage (Fig. 1). We expected post-repair values

**Table 1** Analysis of variance for %DNA in tail with species and treatment as main effects

Source of variation	df1, df2	F	P
Species	5, 662	94.8	<0.0001
Treatment	2, 662	335	<0.0001
Species x treatment	8, 662	38.4	<0.0001



**Fig. 1** Least-square means of %DNA in comet tails of erythrocytes in six species of colubrid snakes across three different treatments. *Blue* Short lived species, *red* long-lived (see Table 2). *A* Control treatment of no damage beyond baseline, *B* damage treatment that demonstrates the inducible DNA damage with 5 min of UV light., *C* damage and repair treatment that allows 10 min of repair following the 5 min of UV damage

significantly lower than induced damage levels. This was observed in all long-lived and one short-lived species. As well, the degree of repair differed among species. Adjusted pair-wise comparisons of least-square means revealed that the three long-lived species repaired a significantly larger fraction of baseline DNA damage than did the three short-lived species (Table 2). These findings are consistent with the hypothesis that long-lived organisms have either better repair mechanisms or more efficient repair than short-lived organisms. A causative role for different types of DNA damage and repair would be the next step to elucidate in this comparative framework.

#### Evolution of lifespan and its correlates within a species of colubrid snake

The main reptile aging research program in my laboratory involves a natural system of garter snakes (*Thamnophis elegans*) that have been continuously monitored for the last 30 years in Lassen County, California. These garter snakes occur in replicate populations of two genetically divergent ecotypes (Bronikowski and Arnold 1999). Ecotype L-fast occurs along lakeshore habitat and exhibits fast

**Table 2** Efficiency of repair expressed as % baseline damage [(B–C)/A]. Formula computed with least square means from the analysis of variance in Table 1

Species	Lifespan (years)	Efficiency (%)
Checkered garter snake ( <i>Thamnophis marcianus</i> )	7	0.30
House snake ( <i>Lamprophis fuliginosus</i> )	9	0.81
Diadem snake ( <i>Spalerosophis diadema</i> )	10	11.1
King snake ( <i>Lampropeltis getula</i> )	30	14.5
Trinket snake ( <i>Elaphe helena</i> )	15	15.3
Corn snake ( <i>Elaphe guttata</i> )	30	22.0

growth, with concomitant early maturation, annual reproduction of large litters, low annual survival and short lifespan. Ecotype M-slow occurs in higher-elevation mountain meadow habitat, and these individuals exhibit slow growth, with concomitant late maturation, infrequent reproduction of small litters, high annual survival and long lifespan. Our previous research has shown that the differences in development rates are due to segregating alleles for fast and slow growth in this system (Bronikowski 2000). At the phylogenetic level, these two ecotypes are indistinguishable based on cytochrome *b* nucleotide sequences (Bronikowski and Arnold 2001). At the population genetic level, neutral microsatellite markers show significant  $F_{st}$  values.  $F_{st}$  (fixation index) is an inbreeding measure that compares the levels of heterozygosity within subpopulations to that in the total (meta-) population. For these populations of garter snakes, significant  $F_{st}$  values demonstrate significant differentiation between L-fast and M-slow populations despite low levels of migration between the two types of populations (Manier and Arnold 2005; Manier et al. 2007). These results support the hypothesis that the two growth and lifespan phenotypes represent evolutionary responses to selection, and potentially local adaptation to habitat differences (Bronikowski 2000). The specific premise relevant here is that. The differences in extrinsic mortality between the two ecotypes have shaped the two life history patterns through natural selection.

Nonterminal procedures to measure and manipulate metabolism, cellular stress responses, and immune function have been undertaken in my laboratory on offspring of these two ecotypes. Our current research is focused on the following questions. Within

ecotype, how do physiological and immune traits covary with age? Between ecotypes, how have the different mortality environments (after Hamilton 1966) shaped relationships among physiology, immune, and life history traits? Within ecotype, how do stress-repair mechanisms impact development and aging? Between ecotypes, how has stress acted as a selective agent to shape the interaction of cellular physiology and life history? Addressing these questions within ecotypes informs us of the current importance of these proximate mechanisms of aging. Comparing the results of these manipulation experiments between ecotypes reveals the relative importance of immune and stress challenges as selective forces (evolutionary mechanisms) in the evolution of aging and places these physiological adaptations in an ecological context. For example, physiological adaptation may release organisms from free-radical production, opening up new evolutionary avenues for longevity in snakes. Alternatively, longevity may itself be adaptive in some ecological contexts, which may then select for release from free radical stress. Our laboratory experimental design on individuals from replicate populations nested within the two life history ecotypes will identify the proximate and ultimate mechanisms of senescence evolution in this system.

Although many of these experiments are ongoing, our completed research (Robert et al. 2008; K.A. Robert and A.M. Bronikowski, unpublished data) offers some unique insights into the relative importance of predation, immune challenge, and starvation resistance in determining the mortality environments in which these snakes have evolved, and reveals the importance of the free radical/repair hypothesis of aging versus other potential mechanisms. First, with respect to the evolution of aging, earlier work demonstrated that these populations experience different degrees of food stress (Bronikowski and Arnold 1999). M-slow snakes have evolved in an unpredictable food-availability environment, in which the likelihood of prey availability is only 50% in any given year. Elsewhere we have hypothesized that a high incidence of zero-food years would be a strong selective force for slow growth, and for channeling energy into reproduction rather than growth in bountiful years (Bronikowski and Arnold 1999). A food-restricted ecology should favor slow growth evolutionarily, which would either directly (through

less physical space) or indirectly (through limiting energy available for vitellogenesis) decrease reproductive output and increase interbirth interval. Neonates from M-slow and L-fast phenotypes, when raised in the common laboratory environment, exhibit their source phenotype of slow and fast growth, respectively. That field patterns are repeated in the laboratory provides evidence that there is a genetic basis specifically to the growth and maturation phenotypes (Bronikowski 2000).

L-fast garter snakes live on average 2 years past maturity if they survive to sexual maturation at 2 years of age. Thus the average L-fast snake, should it survive through the high-mortality neonatal and juvenile stages, has a mean opportunity for reproduction of two events. This pattern contrasts with M-slow snakes, which have an extended immature stage marked by low mortality, followed by an extended adult stage. The average M-slow snake lives for 4 years beyond sexual maturation, which occurs around 4 years of age; thus there is a two-fold difference in mean lifespan of L-fast and M-slow snakes (4 vs 8 years). However, the L-fast females that do not die from predation or disease continue growing and reproducing; they can average three times the reproductive output of M-slow females (Sparkman et al. 2007).

A recent revision of evolutionary aging theory counters the prediction that high-mortality environments will always lead to rapid senescence, phenotypic breakdown, and short life (Williams and Day 2003). Specifically, evolution in high-mortality environments may be expected to lead to long lifespans when the survival of individuals is dependent on condition. To date, Trinidadian guppies (Reznick et al. 2004) and these garter snakes have both provided supporting data for these new, somewhat counterintuitive theoretical predictions.

At the cellular level, results from the same set of assays as applied in the comparative study of colubrid snakes discussed earlier yielded similar findings. For example, neonates from the L-fast ecotype consumed more mass-independent oxygen, had lower mitochondrial ATP:O<sub>2</sub>-consumed ratios, produced more hydrogen peroxide in the electron transport chain, and repaired DNA damage less efficiently than neonates from the M-slow ecotype (unpublished data). Other experiments testing for causative relationships among corticosterone production, immunocompetence, feed-

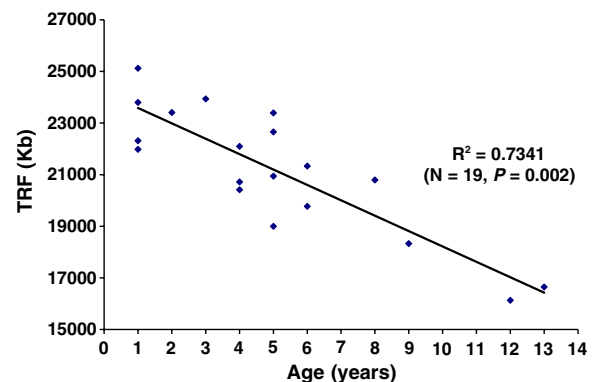
ing behavior, and growth are underway and should advance our understanding of reptilian physiology, as well as provide better biomarkers of aging and immunity in these animals. In addition, because snakes are ectothermic, we can begin to address how cellular physiology and life history affect lifespan by experimentally manipulating metabolic rate.

### Telomere shortening rate

An additional variable that we have measured in the California garter snake system that has been of broad interest in the aging literature is telomere shortening rate. Telomere shortening rate has not been extensively examined in reptiles, but may provide an important comparison with the birds. The literature on avian telomere dynamics suggest that rate of telomere shortening can be predictive of survival and perhaps even lifespan (e.g., Haussmann et al. 2005; reviewed in Monaghan and Haussmann 2006).

#### Telomere shortening rate: methods

We determined telomere restriction fragment (TRF) length from erythrocyte DNA according to the methods of Haussmann and Vleck 2002. For this study, we used male snakes from both ecotypes that ranged in age from newborn through adult. Standard histological sectioning of a tail vertebra was used to count the growth rings and infer age of individuals (for methods, see Wayne and Gregory 1998). The subject males were hibernated in the laboratory over the winter and, upon



**Fig. 2** Telomere restriction fragments for male garter snakes of known age

removal to room temperature in the spring, were allowed to acclimate for 1 week. Fresh whole blood was collected from each snake and immediately diluted in ice-cold 2% EDTA (1:1 dilution). Erythrocyte DNA was extracted using agarose plugs (Biorad, Hercules, CA) and digested using restriction enzymes (*Hae*III, *Hind*III, *Hinf*I). The digested DNA fragments were then separated on a 0.8% non-denaturing agarose gel for 21 h, at 3 V/cm and 14°C. The variability of within-gel DNA migration was determined by running a duplicate sample from one individual in two lanes on each gel. Two <sup>32</sup>P-labeled ladders were also used on each gel to estimate the position and average length of telomeres. The gel was dried and then hybridized for 16 h at 37°C with <sup>32</sup>P-labelled (C<sub>3</sub>TA<sub>2</sub>)<sub>4</sub> oligonucleotides in hybridization solution. We used a phosphor imager system to visualize TRFs, and densitometry was used to determine the position and strength of the radioactive signal in each of the lanes (see Haussmann and Vleck 2002, Haussmann et al. 2003 for detailed protocols).

#### Telomere shortening rate: results

TRFs decreased with age in this set of snakes (Fig. 2). Telomere restriction fragments ranged in size from 16 to 25 kb. They were strongly negatively associated with age (Pearson's  $r = -0.86$ ,  $n = 19$ ,  $P = 0.002$ ). Although this analysis lacked the statistical power to test for an ecotype-by-age interaction, the significantly negative correlation with age (as estimated by number of bone growth rings) suggests that telomere dynamics could either respond to or cause cellular physiology differences between the ecotypes. Further experiments would need to be conducted to confirm whether telomere-shortening rate differs between the long- and short-lived ecotypes of garter snakes. The TRF values are in general agreement with those reported for alligators (27–34 Kb, Scott et al. 2006) and contrast with the surprisingly large values that have been found in erythrocytes of the painted turtle (Paitz et al. 2004: all > 60 kb for animals ranging in age from hatchling to old, mature turtles of 12 years of age).

Increased rate of telomere shortening with immune or oxidative stress in species of reptiles that have highly plastic rates of erythrocyte recruitment is a highly promising direction for reptile aging research to move towards. Furthermore, the use of a suite of dependent variables that are standard measures in

mammalian studies, ranging from telomeres and mitochondrial H<sub>2</sub>O<sub>2</sub> production to DNA damage repair will provide unique insights into the similarities and differences in mammalian and reptilian aging, and may lead to findings of much broader impact when discovered in organisms with very different rates of aging-related deterioration.

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