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Testing the 'free radical theory of aging' hypothesis: physiological differences in long-lived and short-lived colubrid snakes

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Summary

We test the 'free radical theory of aging' using six species of colubrid snakes (numerous, widely distributed, non-venomous snakes of the family Colubridae) that exhibit long (> 15 years) or short (< 10 years) lifespans. Because the 'rate of living theory' predicts metabolic rates to be correlated with rates of aging and oxidative damage results from normal metabolic processes we sought to answer whether physiological parameters and locomotor performance (which is a good predictor of survival in juvenile snakes) mirrored the evolution of lifespans in these colubrid snakes. We measured whole animal metabolic rate (oxygen consumption $\dot{V}_{O_2}$), locomotor performance, cellular metabolic rate (mitochondrial oxygen consumption), and oxidative stress potential (hydrogen peroxide production by mitochondria). Longer-lived colubrid snakes have greater locomotor performance and reduced hydrogen peroxide production than short-lived species, while whole animal metabolic rates and mitochondrial efficiency did not differ with lifespan. We present the first measures testing the 'free radical theory of aging' using reptilian species as model organisms. Using reptiles with different lifespans as model organisms should provide greater insight into mechanisms of aging.

Key words: aging; free radical theory; metabolism; oxidative stress; rate of living theory; reptile; senescence.

Introduction

Aging or senescence is one of the most complex biological processes and is often characterized by deterioration of physical function, including a reduction in fecundity, loss of vitality, and an increase in vulnerability. One of the first theories of aging, the 'rate of living theory' (Rubner, 1908; Pearl, 1928), is based on the observation that species with high metabolic rates exhibit a shorter lifespan than those with low metabolic rates and the rate of metabolism is a function of body size. This inverse correlation between metabolic rate and lifespan is based on the thought that long-lived species are on average bigger and spend fewer calories per gram of body mass than smaller, short-lived species (Kleiber, 1975). Although this is true among many species in the animal kingdom, it does not apply universally (e.g., in birds and bats) (Austad, 1997; Brunet-Rossinni & Austad, 2004).

The rate of living theory was developed further (Harman, 1956) to highlight the role of oxidative stress resulting from normal metabolic processes to explain aging, and this extension is referred to as the 'free radical theory of aging'. Oxidative stress is a strong candidate mechanism of aging (Finkel & Holbrook, 2000). Since oxidative damage from several sources accumulates with age, the free radical theory of aging postulates that aging results from increasing accumulation of damage generated by reactive oxygen species (ROS) (reviewed in Beckman & Ames, 1998) that goes unchecked by antioxidant defenses and cellular repair mechanisms with age. ROS are generated from several sources, although ROS produced by mitochondrial processes has been suggested as the source of damage that results in aging (Barja, 2002a,b; Linford *et al.*, 2005). The largest source of free radicals originates from the process of oxidative phosphorylation, which is the terminal process of cellular respiration that occurs in the mitochondria to generate adenosine triphosphate (ATP). Since ROS are a result of cellular metabolism, the free radical theory of aging provides a mechanism for the rate of living theory (Harman, 1981). This reformulation, which attributes aging to oxidative damage resulting from cellular metabolism rather than to metabolic rate *per se*, accommodates numerous species (e.g. birds, bats, and naked mole-rats) that defy the correlation proposed by the original formulation.

The evolutionary theory of aging is linked to the life history theory in which schedules of reproduction and survival result from a trade-off between investment in somatic maintenance and investment in reproduction (Williams, 1957; Kirkwood, 1977; Kirkwood & Rose, 1991; Austad, 1997). Specifically, animals that experience high mortality rates have lower probability of surviving to their next reproductive bout. Thus, it would be evolutionarily advantageous for these animals to invest in rapid growth and early reproduction instead of somatic maintenance. Animals that experience low-mortality rates can spread their lifetime reproductive output over more bouts (because they have a good chance of surviving long enough) and thus invest in somatic maintenance and greater parental investment in fewer offspring.

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Table 1 Geographic distribution and life history of the six study species

Common name	Geographic distribution	Sexual maturity	Longevity
Common king snake	Widespread USA (except northern states)	3–4 years	25+ years
Corn snake	South-eastern USA	2–3 years	25+ years
Trinket snake	India, Sri Lanka, Nepal and Bangladesh	2 years	15 years‡
Eastern diadem snake	Egypt, West Bengal, India, Sri Lanka and Pakistan	< 2 years	10 years
Checkered garter snake	Southern USA and Mexico	< 2 years*†	7 years
African house snake	Widespread Africa (south of Sahara)	< 1 year	9 years

Age of sexual maturity based on Bartlett & Tennant (2000); N. Ford pers. comm.
*Specific data lacking, age based on related species *Thamnophis sirtalis*, Seigel & Ford (1987); Rossman *et al*. (1996).
†Can have multiple clutches, Ford & Karges (1987).
Longevity is based on captive records (Slaven's longevity index – http://www.pondturtle.com/longev.html; Patnaik (1994); and ‡from www.reptilia.org)

Evolutionary predictions for the extension or reduction in lifespan are specific outcomes of the mortality environment (Charlesworth, 1994). Faster moving individuals may be better able to escape potential predation events, hence locomotor performance is widely used as a measure of fitness and survival (Christian & Tracy, 1981; Jayne & Bennett, 1990; Downes & Shine, 1999; Garland, 1999). For example, locomotor performance in the common garter snake (*Thamnophis sirtalis*) is positively correlated with survival; faster offspring are more likely to survive to their second year than slower offspring (Jayne & Bennett, 1990). Locomotor performance is also positively correlated with survival under natural conditions in squamate lizards (Warner & Andrews, 2002; Miles, 2004) and turtles (Janzen, 1995). Locomotor performance is well retained over time, in that an individual that is relatively fast at one time will remain relatively fast at another. This is important for measuring performance of neonates and inferring future lifetime effects. To date, all species examined have significantly high repeatability of locomotor performance (reviewed in Bennett & Huey, 1990), which supports its use as a proxy for organismal fitness.

Reptiles are ideal taxa to examine metabolism and longevity. Reptiles tend to show a lower rate and intensity of aging especially in comparison to mammals (Patnaik, 1994). Many reptiles have indeterminate growth and increased fecundity with body size, also reproductive senescence is absent in many species (Congdon *et al*., 2001, 2003; Sparkman *et al*., 2007). Reptiles exhibit a wide array of lifespans from short-lived (< 2 years) to extremely long-lived (150+ years) and may exhibit unique mechanisms to delay aging and age-related debilitation making long-lived reptiles an underutilized model in the study of senescence. Reptiles are ectothermic and have the ability to reduce metabolism extensively in response to unfavorable conditions. Some studies suggest that hibernating animals may live longer (Lyman *et al*., 1981; Stuart & Brown, 2005) due to a period of reduced metabolism. Many animals enhance their survival under extreme or unfavorable conditions by entering quiescent states of hibernation, torpor or estivation that are defined by profound reductions in metabolic rate and body temperature. Many reptiles undergo periods of hibernation and can endure periods of extremely reduced metabolism and extended periods of fasting (Secor & Diamond, 1995). The examination of energy metabolism and performance in reptiles with variable rates of aging should therefore provide insights into the biology of aging in general, and specifically, the generality of the free radical theory of aging.

In this study, we measured whole animal physiology (oxygen consumption), locomotor performance (as a measure of fitness and survival), and cellular physiology (mitochondrial oxygen consumption and ROS production) in neonatal snakes to evaluate correlations of these traits between species of short longevity (< 10 years) and those that are long-lived (> 15 years) (Table 1). We hypothesize that longer-lived species will exhibit reduced metabolic rates, increased physical performance (fitness/survival), and more efficient mitochondria that produce reduced amounts of oxidants, in comparison to short-lived species.

Results

Whole animal physiology

Metabolic rates

To determine whether resting metabolic rates differed between short-lived and long-lived colubrid snakes, we measured oxygen consumption using closed system respirometry. Metabolic rates ($\dot{V}_{O_2}$) were strongly influenced by body mass (Table 2). Once the effect of body mass was removed, there was no effect of lifespan on metabolic rate ($\dot{V}_{O_2}$), although there was a significant effect of temperature (Table 2; Fig. 1A). Metabolic rates increased as

Table 2 Repeated measures analysis of covariance for metabolic rates ($\dot{V}_{O_2\,ml\,h^{-1}}$). Mass is the covariate represented as the natural log of body mass

Effect	d.f.	MS	*F*	*P*
Between				
Mass (Ln)	1	25.173	51.26	< 0.0001*
Lifespan	1	0.398	0.81	0.3763
Error	25	0.491		
Within				
Temperature	2	0.190	1.48	< 0.0001*
Temperature * lifespan	2	0.017	0.14	0.9695
Error (temperature)	50	0.129		

MS, mean squares.
*Significant.

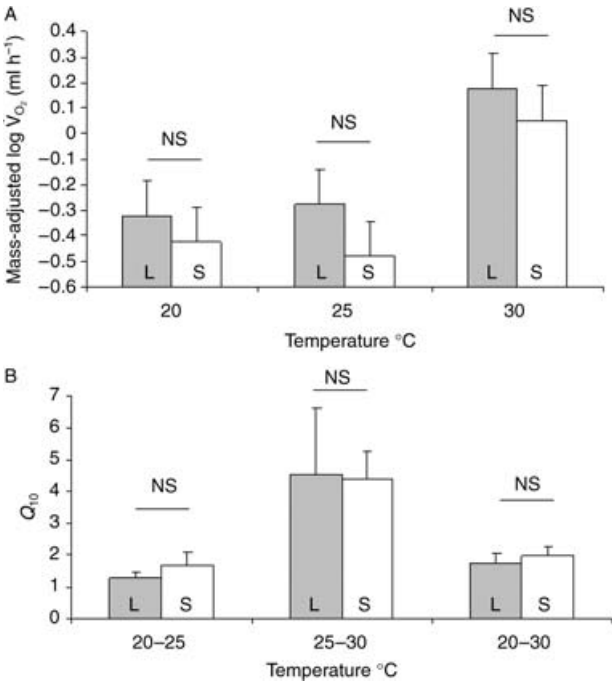

Fig. 1 (A) Mass-adjusted resting metabolic ($\dot{V}_{O_2 ml\,h^{-1}}$) rate at 20, 25, and 30 °C. Shaded bars represent mass adjusted means (analysis of covariance adjusted for individual effects of mass) for long-lived snakes (L) (n = 14) while open bars represent mass adjusted means for short-lived snakes (S) (n = 14). Error bars are ± SE. (B) Q_{10} values calculated to express the temperature dependence of metabolism. Shaded bars represent long-lived snakes (L) (n = 14) while open bars represent short-lived snakes (S) (n = 14). NS, not significant.

temperature increased, and long-lived and short-lived snakes had equal rates of metabolism over the range of temperatures measured.

We measured the Q_{10} (temperature coefficient) of metabolic rates between long- and short-lived species as a measure of the rate of change as a consequence of increasing temperature by 10 °C using the equation: $Q_{10} = (R_2/R_1)^{(10/T2-T1)}$; where R is the rate and T is the temperature. Q_{10} is a standard way to express the temperature dependence of a biological process (Davies & Tribe, 1969), a Q_{10} of 2 indicates that the rate doubles and a Q_{10} of 3 indicates that the rate triples. The thermal sensitivity of metabolic rates was calculated for the 20–25 °C, 25–30 °C, and 20–30 °C temperature intervals separately for each snake. The Q_{10} of metabolic rates did not differ between long- and short-lived species over all the temperature range increases [analysis of variance (ANOVA): 20–25 °C, $F_{1,26} = 0.003$, $P = 0.954$; 25–30 °C, $F_{1,26} = 0.036$, $P = 0.851$; 20–30 °C, $F_{1,26} = 0.299$, $P = 0.589$]. However, Q_{10} values greater than 2 indicated higher thermal dependence of metabolic rates from 25 to 30 °C in both groups. Both long-lived and short-lived species had low thermal dependence of metabolic rates from 20 to 25 °C and from 20 to 30 °C (Q_{10} values ≤ 2) (Fig. 1B). Resting metabolic rates did not correlate with lifespan as would have been predicted by the rate of living theory hypothesis.

Table 3 Repeated measures analysis of covariance for locomotor performance over sprint (1 m) and burst (25 cm) distances

Effect	d.f.	MS	F	P
Sprint (1 m)				
Between				
Mass (Ln)	1	0.023	9.57	0.005*
Lifespan	1	0.056	22.70	< 0.0001*
Error	25	0.002		
Within				
Temperature	2	0.007	10.25	0.0002*
Temperature * mass	2	0.005	7.59	0.0013*
Temperature * lifespan	2	0.005	7.50	0.0014*
Error	50	0.001		
Burst (25 cm)				
Between				
Mass (Ln)	1	0.038	4.44	0.0454*
Lifespan	1	0.115	13.50	0.0011*
Error	25	0.009		
Within				
Temperature	2	0.027	6.69	0.0027*
Temperature * mass	2	0.017	4.25	0.0198*
Temperature * lifespan	2	0.048	11.78	< 0.0001*
Error	50	0.004		

*Significant.

Locomotor performance

Locomotor performance was determined as a measure of fitness and survival. Longer-lived species are expected to show greater locomotor performance equating to increased fitness and survival. The mean speed calculated from three performance trials over 1 m and the fastest 25 cm in any trial were used to determine sprint (1 m) and burst performance (25 cm). One individual snake (diadem snake) refused to move during a trial and therefore mean sprint speed was calculated from the average of two trials. Longevity has a significant effect on locomotor performance (Table 3, Fig. 2), with longer-lived species outperforming short-lived species at both sprint and burst speeds. Temperature and lifespan significantly interacted to affect both sprint and burst speeds (Table 3). The speed of short-lived snakes was fairly insensitive to changes in temperature, whereas warmer temperatures produced faster speeds in long-lived species (Fig. 2). This may reflect wide temperature preferences or activity ranges in the shorter-lived species.

We measured the Q_{10} (temperature coefficient) of locomotor performance between long- and short-lived species as a measure of the rate of change as a consequence of increasing temperature by 10 °C using the same calculation used for metabolic rates described previously. The thermal sensitivity of locomotor speed was calculated for the 20–25 °C, 25–30 °C, and 20–30 °C temperature intervals separately for each snake. The Q_{10} of sprint speed over 1 m between long- and short-lived species did not differ between 20 and 25 °C (ANOVA: $F_{1,27} = 3.245$, $P = 0.083$) and between 25 and 30 °C (ANOVA: $F_{1,27} = 3.239$, $P = 0.084$), but burst speeds over 25 cm in long- and short-lived snakes were more thermally sensitive over both temperature

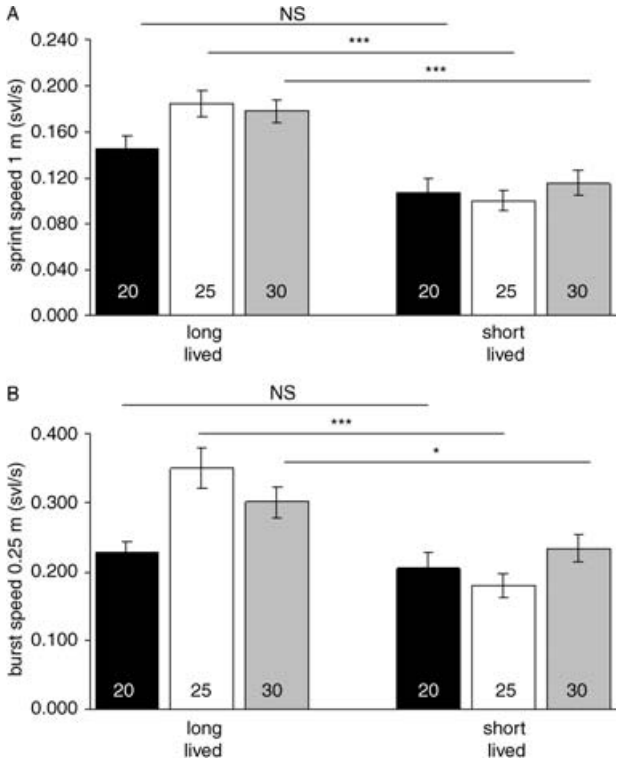

Fig. 2 Locomotor performance in long- (n = 14) and short-lived (n = 14) Colubrid snakes at 20, 25, and 30 °C over (A) 1 m sprint distance and (B) 25 cm burst distances. Black bars represent 20 °C, white bars represent 25 °C, and grey bars represent 30 °C. Speed measured in body lengths (SVL – snout-vent length) per second. NS, not significant, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$.

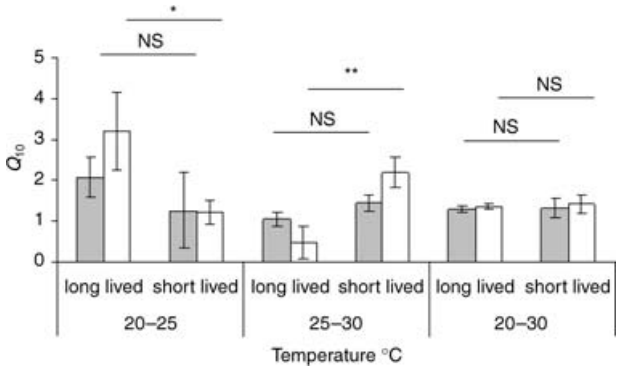

Fig. 3 Q_{10} values calculated to express the temperature dependence of locomotor performance. Shaded bars represent sprint speeds over 1 m while open bars represent burst speeds over 25 cm. NS, not significant, $*P < 0.05$, $**P < 0.01$.

ranges, 20–25 °C (ANOVA: $F_{1,27} = 6.938$, $P = 0.014$) and 25–30 °C (ANOVA: $F_{1,27} = 10.860$, $P = 0.003$) (Fig. 3). The Q_{10} of sprint and burst speeds between long- and short-lived species did not differ between 20 and 30 °C (ANOVA: sprint, $F_{1,27} = 0.002$, $P = 0.958$; ANOVA: burst, $F_{1,27} = 0.078$, $P = 0.782$).

Longer-lived snakes show greater aggression following a trial than short-lived snakes (G-statistic: 21.3, $P < 0.0001$) and short-lived snakes were more likely to attempt to change direction during a trial than longer-lived species (G-statistic: 12.9, $P < 0.0001$) (Fig. 4). Antipredator behaviors and locomotor performance may play an important role in extrinsic mortality rates and hence would be expected to correlate with rates of senescence.

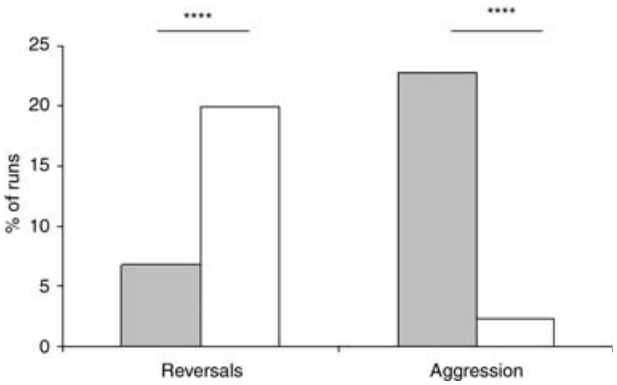

Fig. 4 Percentage of locomotor trials where snakes behaved with quick reversals of direction during the trial or showed aggressive behavior following a trial. Shaded bars represent long-lived colubrid snakes (L) and open bars represent short-lived snakes (S). Behavior is dependent upon lifespan (G-statistic: reversals G = 12.9, aggression G = 21.3), $****P < 0.0001$.

Cellular physiology

Mitochondrial oxygen consumption

Approximately 85–90% of the cellular oxygen is consumed by mitochondria in the process of generating ATP (reviewed in Harper *et al.*, 2004). Efficiency of mitochondrial function is reflected in the respiratory activity of the mitochondria. Neither respiratory control ratios (RCR), state IV oxygen consumption, or mitochondrial efficiency (P : O ratio) differed between long- and short-lived species (Fig. 5A–C). Mitochondria from long- and short-lived species are of equal health (RCR, Fig. 5A) (ANOVA: $F_{1,4} = 0.426$, $P = 0.275$), consume oxygen at equal rates in state IV (Fig. 5B) (ANOVA: $F_{1,4} = 0.407$, $P = 0.268$) and convert a known amount of adenosine diphosphate (ADP) to ATP with equal efficiency (P : O ratios) (Fig. 5C) (ANOVA: $F_{1,4} = 0.186$, $P = 0.345$)

Mitochondrial ROS production

We measured ROS produced by mitochondria using a fluorometric assay that detects the rate of mitochondrial H_2O_2 production (Barja, 1998, 2002b). Short-lived species produced significantly greater amounts of mitochondrial ROS than long-lived species (Fig. 5D) (ANOVA: $F_{1,4} = 6.570$, $P = 0.031$). This supports the generalization that lifespan is negatively correlated with rates of ROS generation.

Discussion

In this study, whole animal resting metabolic rates did not correlate with lifespan as expected under the rate of living theory model, and both lifespan groups consumed oxygen at relatively equal rates over the temperature ranges measured. Low Q_{10}

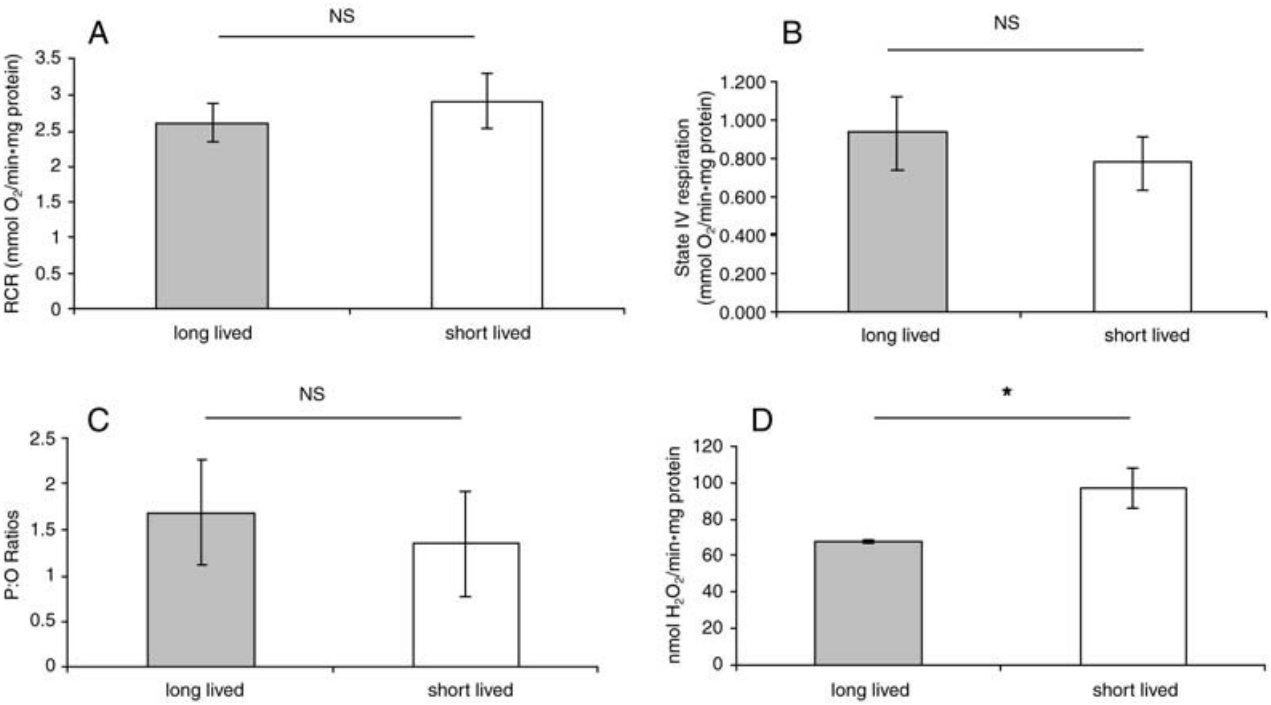

Fig. 5 Cellular physiology in long-lived (shaded bars) (n = 3) and short-lived (unshaded bars) (n = 3) colubrid snakes at 28 °C. (A) RCR values = state III respiration/state IV respiration. (B) Mitochondrial oxygen consumption in state IV (with succinate as substrate). (C) P : O ratios (amount of ATP produced/O_2 atom). (D) Mitochondrial ROS production. All presented as means ± SE. NS, not significant, $^*P < 0.05$.

values indicated that body temperature changes can occur without much metabolic cost, which may reflect wide temperature activity ranges for the snakes in the study. Each species in this study occurs over a wide geographical range, so although specific data on preferred temperatures have not been gathered, presumably each species must respond to a wide distribution of active thermal environments. Despite the broad acceptance of the rate of living hypothesis, there has been little evidence that metabolic rates influence senescence in endotherms (Trevelyan *et al.*, 1990; Harvey *et al.*, 1991) or ectothermic invertebrates (Van Voorhies *et al.*, 2004). Several studies have cast doubts on the predicted correlation of metabolic rates with longevity and senescence. For example, some rats and mice with higher metabolic rates can live longer than those with lower rates (Holloszy & Smith, 1986; Speakman *et al.*, 2004). A point of debate is whether species that undergo periods of hibernation, diapause or torpor can live longer than nonhibernating species. Species that undergo periods of hibernation can decrease metabolic rates significantly (Miller & Hadfield, 1990; Clegg *et al.*, 1996; Geiser, 2004). To date results are varied; however, some reports have shown that species that enter periods of hibernation may live longer (Lyman *et al.*, 1981; Tatar & Yin, 2001; Brunet-Rossinni & Austad, 2004), which may suggest that a period of metabolic reduction could increase lifespan. It would be of interest to measure metabolic rates in the snakes from this study during periods of extreme low temperatures to mimic hibernating temperatures and then again at arousal from hibernation to establish if there are differences in increased costs of arousal between the two lifespan groups.

Locomotor performance differed between long- and short-lived species in this study with long-lived species characterized by faster sprint and burst speeds, and thus, overall enhanced performance. Locomotor capacity significantly predicts survivorship (Jayne & Bennett, 1990; Downes & Shine, 1999; Le Galliard *et al.*, 2004) and dominance in reptiles (Garland *et al.*, 1990) and so serves as a proximate measure of a potentially important component of survival in the wild. In addition, antipredator behavior may be an important factor influencing locomotor speed and hence survivorship in neonatal snakes. Short-lived species were more likely to reverse and change direction than longer-lived species, and longer-lived species were more likely to show aggressive behavior on completion of locomotor trials than short-lived species. Reversals of direction have been associated with snakes that rely upon crypsis to avoid detection by predators, so that when they have been detected they abruptly change direction to return to a cryptic position (Brodie, 1989). Two of the longer-lived species (common king snake – *Lampropeltis getula*, and trinket snake – *Elaphe helena*) exhibited 'warnings' when pursued along the racetrack: *L. getula* used vigorous tail rattles and *E. helena* puffed up and flushed their skin red. On completion of runs these snakes often behaved aggressively with mouth gapes, rearing up and striking when re-approached. Longer-lived species may not only be physiologically better prepared to sustain life but also better equipped behaviorally and defensively to ensure a greater chance of survival. Evolutionary hypotheses predict that species that are better protected to avoid extrinsic mortality should live longer (Charlesworth, 1994). For example, flying mammals can live up to three times

longer than nonflying mammals of the same size (Austad & Fisher, 1991), social mammals protected by group defense are longer-lived (Sherman & Jarvis, 2002), and venomous species or those with chemical protection have been shown to have longer maximum lifespans than nonprotected species (Blanco & Sherman, 2005). Life history and behavior are therefore important traits to consider in the avoidance of predation and the evolution of senescence.

The free radical theory of aging was supported at the cellular level with longer-lived species exhibiting lower ROS production than short-lived species. This is consistent with longer-lived species investing more in somatic maintenance through specific mechanisms that decrease electron leakage in mitochondria. The mitochondrial production of ROS has been integrated with the rate of living theory to predict that lower mitochondrial activity and/or ROS production should be associated with prolonged life (Barja, 2002a; Sohal, 2002). Oxidative damage determines the lifespan of *Drosophila* under normal metabolic conditions and under conditions of environmental stress (Fleming *et al*., 1992) although another study did not support a correlation between lifespan and ROS production (Miwa *et al*., 2004). Low hydrogen peroxide production and slightly higher P : O ratios in mitochondria from longer-lived snakes, despite similar whole animal metabolic rates, supports the contention that whole animal metabolism is not always proportional to the rate of aging as predicted by the rate of living theory hypothesis. These results additionally raise the possibility of structural and functional differences between the mitochondrial populations of the two lifespan groups that result in reduced ROS production and/or reduced accumulation of oxidative damage. Besides increasing sample sizes in mitochondrial function measures, future lines of research should include examination of antioxidant defense systems (e.g., superoxide dimutase, glutathione, and catalase), assessment of accumulated oxidative damage over time, and evaluation of the activity of repair mechanisms.

Energy metabolism and longevity within a species or groups of species have been studied using various methods (transgenic or mutant models, different strains, individual variation within a species, environmental manipulation, and caloric restriction) with very different patterns of association from positive to negative, and those of no significance (reviewed in Speakman, 2005). Comparisons have been made across different strains of dogs and mice that suggest a positive association of metabolism and longevity (Speakman *et al*., 2002, 2003). In contrast, however, several studies of different strains of *Drosophila* show no association of these traits between the strains (Promislow & Haselkorn, 2002; Van Voorhies *et al*., 2003). There are relatively few studies utilizing the tremendous individual variation (within species) of energy expenditure, despite the advantages of this comparison (Austad, 1996). One such study of individual variation in a mouse shows a positive effect of metabolism on lifespan (Speakman *et al*., 2004). Testing differences between very different taxa may not be as informative as testing within species with genetically based variation in longevity. To address this concern, current work within our laboratory is examining physiological and extrinsic differences within the western terrestrial garter snake, *Thamnophis elegans*, a species of colubrid snake with both long- and short-lived genotypes in nature (Bronikowski & Arnold, 1999; Bronikowski, 2000).

In conclusion, whole animal metabolic rates did not support the rate of living theory model and did not correlate directly with longevity. However, our results support the free radical theory of aging and hence, the rate of living theory at the cellular level, to provide some explanation for the differences in lifespan among the species examined. Thus, we present the first measures testing the free radical theory of aging using reptilian species as a model. There is considerable scope for further research and refinement of our study. Inclusion of very short-lived species (< 2 years) may help distinguish more subtle differences by examining a comparison of long-lived (25+ years) and short-lived species (< 2 years). Also, the examination of differences in lifespan and physiology within a species should prove valuable, for example our work on *T. elegans*. It is clear that more in-depth studies using reptile species with various longevities and energy requirements as model organisms should provide greater insight into mechanisms of aging.

Experimental procedures

Study animals

Data were obtained from 26 captive bred neonatal (< 6 months) colubrid snakes from second-generation laboratory-reared mothers of six species [(*L. getula* (*n* = 6)*, Elaphe guttata* (*n* = 5), *E. helena* (*n* = 3), *Spalerosophis diadema* (*n* = 6), *Thamnophis marcianus* (*n* = 3), *Lamprophis fuliginosus* (*n* = 5) obtained from the Ophidian Research Colony (University of Texas, Tyler, TX, USA). Three species are long-lived (15+ years) and three have short longevity (< 10 years) (Table 1). These species were chosen to represent evolutionarily independent origins of longevity so that species-specific longevity does not follow phylogenetic groupings and any interspecific correlations between traits cannot be attributed to phylogenetic conservatism. The most recent phylogenetic hypothesis for colubrid snakes suggests all study species are monophyletic groups and differences in longevity will be highly labile within the group (Fig. 6).

The common king snake (*L. getula*) is widespread throughout the USA, diurnally active and long-lived. Corn snakes (*E. guttata*) are also long-lived but largely nocturnal although can be active during the day. Trinket snakes (*E. helena*) are widely distributed throughout India, Sri Lanka, Nepal, and Bangladesh, where they are diurnally active and long-lived. Eastern diadem snake (*S. diadema*) occurs throughout Egypt, West Bengal, India, Sri Lanka, and Pakistan; they are diurnally active and have short lifespans. The checkered garter snake (*T. marcianus*), occurs throughout southern USA, has a short lifespan and is largely diurnally active, although it can be nocturnal in ideal conditions. African house snakes (*L. fuliginosus*) are widespread throughout Africa; they are primarily nocturnal and have short longevity. Body sizes and weights for snakes in the study are shown in Table 4.

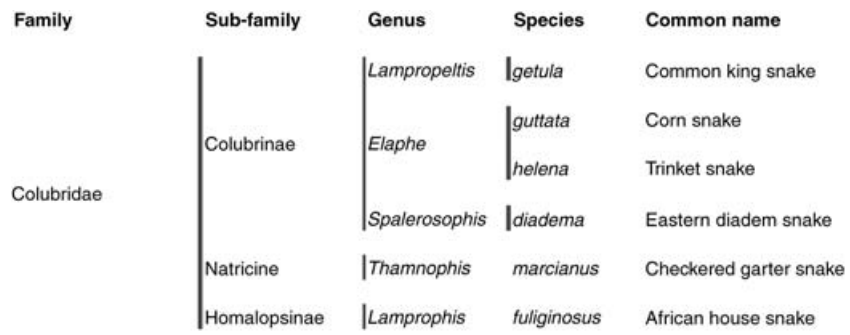

Fig. 6 Phylogenetic relationship of the six species obtained for the study from the Ophidian Research Colony (University of Texas, Tyler, TX, USA) (Zug *et al.*, 2001).

Table 4 Body sizes of neonatal snakes for which measurements were made

		Snout–vent length (mm)		Mass (g)	
Species	*n*	Mean (SE)	Range	Mean (SE)	Range
Lampropeltis getula (common king snake)	6	368.5 (8.18)	340–386	13.24 (1.30)	8.52–16.08
Elaphe guttata (corn snake)	5	296.4 (6.32)	273–307	5.51 (0.34)	4.74–6.34
Elaphe Helena (trinket snake)	3	290.7 15.38	260–308	7.86 (1.02)	5.89–9.31
Spalerosophis diadema (diadem snake)	6	344.3 (3.57)	330–386	10.49 (0.13)	10.16–11.01
Thamnophis marcianus (checkered garter Snake)	3	277 (44.03)	230–365	9.64 (4.18)	5.09–17.99
Lamprophis fuliginosus (African house snake)	5	214 (5.18)	200–232	3.28 (0.20)	2.95–9.31

Whole animal physiology

Metabolic rates

Resting metabolic rates ($\dot{V}_{O_2}$) were measured using closed system respirometry (Vleck, 1987), for each snake at three trial temperatures (20, 25, and 30 °C) to encompass preferred body temperatures of all species. Body temperature is one of the most important ecophysiological variables affecting reptilian function (Huey & Slatkin, 1976). Very little is known of the natural history of all six species; therefore, a range of temperatures was chosen. Snakes were placed within metabolic chambers in incubators the night prior to trials to become accustomed to test conditions (Hare *et al.*, 2004). Metabolic chambers consisted of darkened aluminum cans sealed with modified lids to include a tube with a stop cock on the end. Incubators were set to trial temperatures at least 2 h before trial commencement to allow snakes to acclimate to trial temperatures. Prior to the start of each trial, 5 mL of water was injected into each can to ensure saturated air; 50 mL of room air was drawn with a 60-mL syringe and placed within incubators at the trial temperature. At the start of trials this volume of air was injected into the cans, mixed by plunging and withdrawing the syringe twice then 30 mL of air was removed from the can (this sample was used to determine the initial oxygen concentration within the can). Stop cocks were then sealed. At the end of 1 h, 30 mL of air was removed from each can to determine final oxygen concentration. Each can was fit with a thermocouple thermometer and temperature was measured at 15 min intervals to ensure can temperatures met set incubator temperatures. Air pressure was also noted at the start of each trial to calculate oxygen consumption and to allow correction to standard temperature and pressure.

Air samples (both initial and final) were measured for oxygen concentration using an AMATEK N-37M oxygen sensor and AMATEK S-3A/11 oxygen analyzer (Pittsburg, PA, USA). Air samples were injected through columns of water (Drierite) and CO_2 (Ascarite) absorbents before entering the oxygen sensor. The rate of oxygen consumption was determined by the method of Vleck (1987).

Oxygen consumption ($\dot{V}_{O_2}$) was calculated as:

$$\frac{\text{Can volume} * (\text{initial } O_2 \text{ concentration} - \text{final } O_2 \text{ concentration}) * \text{duration}}{(1 - \text{final } O_2 \text{ concentration})}$$

We used analysis of covariance to investigate the effect of lifespan on metabolic rates ($\dot{V}_{O_2}$). Mass was included as a covariate in the analysis since expressing physiological data as a ratio (by dividing by mass) does not always adequately remove the confounding effects of body size (Packard & Boardman, 1999).

Most aspects of behavior and physiology in reptiles are sensitive to body temperature, and therefore the thermal sensitivity of metabolic rates (Q_{10} = metabolic rate at higher temperature divided by metabolic rate at lower temperature) was calculated for the 20–25 °C, 25–30 °C, and 20–30 °C temperature interval separately for each snake. Analysis of variance was used to investigate the effect of lifespan on the metabolic cost of temperature change.

Locomotor performance

For performance trials each snake was run three times at each temperature with a 20-min break between runs. Snakes were tested over the same temperature ranges as metabolic measures (20 °C, 25 °C, and 30 °C) by placing snakes within incubators set at the test temperature. Prior to tests snakes were allowed

a minimum of 2 h to acclimate to the test temperature. All measurements were on a linear racetrack measuring 4 cm wide and 1.2 m in total length. Photocells located at 25-cm intervals along the racetrack recorded the cumulative time taken for snakes to cross each successive infrared beam (to give a total for a 1-m distance) and the fastest speed over any 25-cm interval. The surface of the track consisted of rough sand paper to facilitate locomotion. To begin a trial, an individual was transferred directly from its container to the holding area of the racetrack (first 10 cm prior to first photocell), whereupon it was released and allowed to race a 1-m distance; if necessary, it was chased with an artist's paintbrush with light taps to the tail. We also recorded individuals that behaved aggressively (mouth gape, strike), stopped, or reversed (turned back) and then continued. Those individuals that refused to move were excluded from trials.

The thermal sensitivity of locomotor performance (Q_{10} = speed at higher temperature divided by speed at lower temperature) was calculated for the 20–25 °C, 25–30 °C, and 20–30 °C temperature interval separately for each snake. Analysis of variance was used to investigate the effect of lifespan on the thermal dependence of locomotor performance.

Cellular physiology

Mitochondrial extraction

Snakes were euthanized by rapid decapitation and livers were extracted and transferred to ice-cold isolation buffer containing 250 mM sucrose, 5 mM Tris Base, 2 mM EGTA, pH 7.4. Livers were pooled within species to harvest sufficient mitochondria giving three long-lived and three short-lived samples for all cellular physiology measures. Mitochondria were isolated from liver tissue by differential centrifugation (Palloti & Lenaz, 2001; Pon & Schon, 2001). Briefly, liver tissue was homogenized in isolation buffer using a 100-mL Kontes glass homogenizer (Vineland, NJ, USA). The homogenate was transferred into 50 mL polypropelene centrifuge tubes and was centrifuged in a Thermo IEC ultracentrifuge at 4 °C for 3 min at 1047 ***g***. The supernatant was then transferred to fresh centrifuge tubes and centrifuged at 11 621 ***g*** for 10 min at 4 °C. The supernatant was then discarded and the mitochondrial pellet was gently resuspended with a glass rod. Mitochondrial isolate was transferred to a microcentrifuge tube and remained on ice. A Bradford protein determination was performed on a 1 : 100 dilution of the isolate to ensure mitochondrial concentrations of > 30 mg mL^{-1}.

Mitochondrial oxygen consumption

Respiratory activities of liver mitochondria were measured by determining oxygen consumption in airtight chambers at 28 °C, using a Clark-type oxygen electrode (Hansatech, Norfolk, UK) according to established procedures (Brand *et al.*, 1993; Trounce *et al.*, 1996; Herrero & Barja, 1997). Briefly, succinate was used as the respiratory substrate in incubation medium containing 145 mM KCl, 3 mM $MgCl_2$, 5 mM KH_2PO_4, 30 mM HEPES, 0.1 mM EGTA, 0.1% defatted BSA, pH 7.4. Mitochondrial oxygen consumption in state III and state IV was calculated as:

$$\text{nmols } O_2/\text{min} * \text{mg protein} = \frac{(\text{sample slope} - \text{drift slope})}{2 \times \text{mitochondrial protein in sample}}$$

Respiratory control ratios were calculated as ratio of state III (in presence of ADP) to state IV (after ADP is converted to ATP) rates of respiration.

The amount of ATP produced per oxygen atom was calculated as:

$$\text{P:O} = \text{ATP formed}/O_2 \text{ consumed} = \frac{(0.025 * \text{amount ADP added})}{(\text{Initial } O_2 - \text{final } O_2)/1000}$$

A greater P : O ratio indicates greater mitochondrial efficiency or that a reduced amount of oxygen is consumed to produce a given amount of ATP.

Mitochondrial ROS production

The ROS produced by mitochondria were measured fluorometrically by the method of Hyslop & Sklar (1984; see also Barja, 1998, 2002b), a method based on the coupled oxidation of *p*-hydroxyphenylacetic acid (PHPA) and reduction of hydrogen peroxide (H_2O_2) by horseradish peroxidase. Briefly, the reaction (total volume of 1.5 mL) consisted of incubation buffer (145 mM KCl, 3 mM $MgCl_2$, 5 mM KH_2PO_4, 30 mM HEPES, 0.1 mM EGTA, 0.1% defatted BSA, pH 7.4), mitochondria (0.33 mg ml^{-1}), 0.33 mM PHPA, six units of horseradish peroxidase per mL, 100 units of superoxide dimutase per mL, 0.5 μM rotenone, 2 μM antimycin A, and 5 mM succinate as substrate, which was added to start the reaction. The inhibitors rotenone and antimycin A were included in the reaction to assay maximum rates of H_2O_2 production at Complex III. After 15 min of incubation at 28 °C the reaction was stopped by transferring samples to an ice bath and the addition of 0.5 mL of reaction stopper (0.1 M glycine, 25 mM EDTA, 0.1 M NaOH, pH 12). The rate of H_2O_2 generation was measured as an increase in fluorescence at an excitation maximum of 320 nm and emission maximum of 400 nm using a Hitachi F-2000 fluorescence spectrophotometer (Hitachi High Technologies, Tokyo, Japan). Known concentrations of H_2O_2 generated in parallel were used to construct a standard curve.

Statistical analysis

Data were tested for normality and homogeneity of variances to meet the assumptions of parametric testing prior to analysis; data that failed to meet assumptions were natural log transformed. Tests performed at different temperatures (metabolic rates, $\dot{V}_{O_2}$ and locomotor performance) were analyzed using repeated measures analysis of covariance with lifespan as the factor, temperature as the repeated measure, and mass as the covariate. The other dependent variables: RCR, state IV respiration, P : O, and ROS were analyzed with one-tailed ANOVAs with lifespan as the factor (JMP statistical package, SAS Institute Inc., Cary, NC, USA). All tests were performed to show < 0.05 significance level.

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