Integrative and Comparative Biology, volume 50, number 5, pp. 880–887
doi:10.1093/icb/icq132

SYMPOSIUM

Metabolism, Body Size and Life Span: A Case Study in Evolutionarily Divergent Populations of the Garter Snake (*Thamnophis elegans*)

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From the symposium "Metabolism, Life History and Aging" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2010, at Seattle, Washington.

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Synopsis We present a case study of metabolism, life history and aging in the western terrestrial garter snake (*Thamnophis elegans*). Early research in the field supported the rate-of-living hypothesis as an explanation of aging, which was based on an apparent negative relationship between mass-specific metabolic rate and lifespan in endotherms. This hypothesis in its original form has not withstood additional tests and comparisons between the two main lineages of endotherms—birds and mammals, but there is still much to be discovered of the causative links among rate of oxygen consumption, physiology and life history, particularly in ectothermic reptiles. We present data that show adult short-lived snakes, from naturally occurring ecotypes of garter snakes, have higher mass-specific resting metabolic rates at any given body mass (metabolic intensity) across a series of normal activity temperatures (15–32°C). The short-lived ecotype in this geographic region reaches a larger body size, and has life-history traits that place it at the fast end of a pace-of-life continuum (fast growth, early maturation, high reproductive output) relative to individuals of the small-bodied long-lived ecotype. The difference between ecotypes in metabolic intensity, even after acclimation to identical conditions, may reflect evolutionary divergence and genetic differences between ecotypes. The difference in metabolic intensity is not, however, present at birth, so an alternative is that developmental environment may permanently influence metabolic rate and life history. Such developmental canalization could lead to altered gene expression via environmental influences on the epigenome and result in altered metabolic trajectories in the snakes' natural habitats.

Introduction

Comparative studies, primarily across endothermic species, that relate life span to physiology have long been a fruitful research area in comparative biology (e.g., Rubner 1908; Pearl 1928; Kleiber 1975; Austad and Fisher 1991; Speakman 2005a). Specifically, the rate-of-living hypothesis of longevity (Pearl 1928) proposed that high basal metabolic rates result in short life expectancies, and low metabolic rates result in longer life. The envisioned mechanism was based on an assumption of a finite lifetime energy allotment, that could be used up quickly (high metabolic rates) or slowly (low metabolic rates) (see also Austad this volume). Within any given species, few general rules have emerged on how physiology underpins longevity despite state-of-the-art techniques brought to bear on this question (e.g., the development of transgenic or mutant lines and environmental manipulations such as caloric restriction or exercise). In endothermic vertebrates, these have produced reports of disparate patterns, and even the lack of an association, between metabolism and life span (reviewed by Speakman 2005a). Fewer comparisons of lifespan and metabolic rate have been published among ectothermic vertebrates (e.g., Robert et al. 2007), yet are of interest because ectotherms have the complexity of highly plastic physiological traits—including metabolic rate—that can dramatically fluctuate within individuals over days, seasons, and across an individual's lifetime. This plasticity of metabolic processes may disassociate or otherwise alter the relationship between

Advanced Access publication September 24, 2010

physiology and life-history traits, particularly life span.

Oxygen consumption *per se* (i.e., metabolic rate) does not directly alter rate of senescence and/or life span, but metabolic rate is clearly correlated with life-history traits such as growth rate, and behavioral traits such as dispersal. Thus, metabolic rate continues to be of interest to researchers studying senescence and life span. The widely-studied free-radical/oxidative-stress hypothesis of aging (reviewed by Finkel and Holbrook 2000) offers a mechanism for how metabolism modulates life span. Variation in how oxygen consumption manifests as oxidative damage via production of reactive oxygen species (ROS), and an organism's defenses against such damage, are hypothesized to underlie the phenomenon of aging (Harman 1956). Tests of how organisms of differing life spans counter damage to DNA, proteins, and lipids; and how such variation in defenses relates to life span and rate of aging, comprise a very active area of empirical research that seeks to link metabolism and physiology with life span (e.g., Bronikowski 2008; Ungvari et al. 2008; Monaghan et al. 2009).

Here we present a case study of the western terrestrial garter snake (*Thamnophis elegans*) in which we quantify metabolic rate—a temperature-sensitive trait in reptiles—in snakes that have evolved different life spans. This species of garter snake has been the topic of research on the evolutionary ecology of life-span variation for the past 20 years. Elsewhere we have reported on two ecotypes of this species: a short-lived form with concomitant fast growth, early reproduction, and high reproductive output; and a long-lived form with slow growth, delayed maturation, and low reproductive output. Observations of evolutionarily divergent life histories (Bronikowski and Arnold 1999; Bronikowski 2000), endocrine function (Robert et al. 2009; Sparkman et al. 2009), immune function (Sparkman and Palacios 2009; Palacios et al. 2010), and somatic repair mechanisms (Bronikowski 2008; Robert and Bronikowski 2010) have demonstrated robust differences between the cellular physiologies of these two ecotypes along all of these axes. Many of the differences are in a direction consistent with the hypothesis that more efficient mechanisms of repair and defense are correlated with longer life span (Table 1). We now provide data to test whether total metabolic rate and mass-specific metabolic rate (metabolic intensity) in mature animals raised in their natural habitats differ between the two ecotypes in a direction consistent with the rate-of-living hypothesis. The rate-of-living hypothesis predicts that, all other things being equal, the short-lived ecotype should use energy faster, and consequently have a higher metabolic intensity than does the long-lived ecotype. Because the short-lived ecotype grows faster and to a larger adult body size than the long-lived ecotype, however, it is essential to control for the effects of body size in testing this prediction. We provide the metabolic measurements over a range of body sizes necessary to test this prediction, and comment on other putative mechanisms of aging that have been examined in this model system for life-history evolution.

Materials and methods

Study populations and subjects

The natural populations of western terrestrial garter snakes under study are located in and around Eagle Lake, Lassen County, California. These specific populations form a traditional metapopulation of local sub-populations, separated by significant genetic differentiation (*Fst*), but connected by occasional gene flow through migration, and with local extinctions and recolonizations (Manier et al. 2007). The entire system of populations are all of the Sierran subspecies of *T. elegans* (*T. e. elegans*) (Bronikowski and Arnold 2001), and at least one of the M-slow populations is a source population for this metapopulation (Manier and Arnold 2005). Within this geographic area there are two ecotypes of garter snake that form two distinct and genetically divergent life-history phenotypes. Numbering more than 35 populations, this system can be divided into lakeshore (''L-fast'') ecotypic individuals that grow fast, mature young, annually devote enormous energy to reproduction, and die young; and meadow (''M-slow'') ecotypic individuals that grow slowly, mature late, have small litters every third year (at most), and are long lived (Bronikowski and Arnold 1999; Bronikowski 2000). Interestingly, the direction of gene flow is from M-slow to L-fast locales, which allows tests of the question as to the circumstances under which a fast pace-of-life with short lifespan evolves from a slow pace-of-life. The putative sources of differences in mortality between L-fast and M-slow habitats are a higher abundance of avian predators at lakeshore habitats on one hand (A. M. Sparkman et al., manuscript in review), and differences in resource availability on the other (D. A. Miller et al., manuscript in review), both of which are demonstrably potent sources of selection. Overall, abundant phenotypic plasticity persists for growth, and indeed all of the life-history characteristics, despite their natural placement on a slow-to-fast

Table 1 Characteristics of long-lived and short-lived garter snakes from Eagle Lake, CA (after Schwartz & Bronikowski 2010)

Trait	Long-lived ecotype	Short-lived ecotype
Habitat[a]		
Substrate	Grassy meadow	Rocky lakeshore
Elevation	1630–2055 m	1555 m
Summer daytime temperature	15–30°C	20–34°C
Preferred body temperature (non-gravid)	28°C	28°C
Avian Predators[b]	Medium-bodied raptors	Large-bodied raptors
Food/water availability	Variable across years	Continuous
Major prey types	Anurans, leech	Fish, leech (anurans in flood years)
Common parasites	Tail trematodes	Mites
Morphology/life history[c,d]		
Color and stripe patterns	Black with bright yellow stripe	Checkered, muted grays, browns
Mean adult body size	538 mm (range: 370–598)	660 mm (range: 425—876 mm)
Female maturation size/age	400 mm/5–7 years	450 mm/3 years
Reproductive rate	Infrequent; resource dependent	Annual
Mean litter size	4.3 liveborn (range: 1–6)	8.8 liveborn (range: 1–21)
Newborn mass	2.85 g (range: 1.8–3.5 g)	3.27 g (range: 2.5–4.2 g)
Annual Adult Pr(survival)[e]	0.77 $year^{-1}$	0.48 $year^{-1}$
Median life span (years)	8	4
Physiology[f,g,h,i]		
Mean Metabolic Rate (ml $O_2\,h^{-1}$) at 28°C at 1 month age, both sexes	0.52 (mean mass = 2.62 g)	Statistically equivalent
Mean Metabolic Rate (ml $O_2\,h^{-1}$) at 28°C for adult males	2.09 (mean mass = 21.6 g)	3.12 (mean mass = 22.1 g)
Metabolic Allometry	$MR \propto Mass^{0.59\pm0.06}$	Statistically equivalent
H_2O_2 production under stress	56 pmol min^{-1} (mg mitochondria)$^{-1}$	240 pmol min^{-1} (mg mitochondria)$^{-1}$
DNA repair efficiency to UV damage (%)	73	35
Field baseline corticosterone levels	50 ± 8 ng mL^{-1} plasma	7.7 ± 12 ng mL^{-1}plasma
IGF-1 levels	Lower; resource dependent	Consistently higher
Innate immune response	Low: natural antibodies, complement-mediated lysis, and bactericidal competence	Higher for all three immune measures

[a]Bronikowski and Arnold 1999.
[b]A. M. Sparkman et al., manuscript in review.
[c]Bronikowski 2000.
[d]Sparkman *et al.* 2007.
[e]D. A. Miller et al., manuscript in review.
[f]Sparkman and Palacios 2009.
[g]Robert and Bronikowski 2010.
[h]Sparkman et al. 2009.
[i]This report; MR = metabolic rate.

pace-of-life continuum (Bronikowski and Arnold 1999; Sparkman et al. 2007).

For this particular report, individuals from three L-fast and four M-slow populations were assayed. Fifty-five adult males were sampled from these seven focal populations of western terrestrial garter snake in the summer of 2004. Adult males were used in this experiment to eliminate variation in reproductive status that is present in adult females. These populations have been monitored by mark/recapture from 1976 to the present (Arnold 1992). From analyses of these long-term data, we have seen consistency across this time period in the ecotypes' patterns of survival, reproduction, and life span, despite smaller-scale annual variation (D. A. Miller et al., manuscript in review).

Animals were held in the laboratory for 1 year to normalize to the same body condition. During this time, snakes were maintained individually in

$9 \times 13 \times 8''$ plastic boxes at 28°C on 12:12 L:D cycle. They were fed twice weekly a diet of feeder goldfish until satiation. Snakes were maintained in this manner from August through November (2004) and again, from April–June (2005); snakes were kept at 5°C without lighting from December through March to mimic their normal hibernation period. Snakes were fasted for the month of July prior to the start of this experiment.

Measurement of metabolic rate

Our goal was to assess whether resting metabolic rate (RMR) and mass-specific RMR differed between the long-lived and short-lived snakes in adult animals that had developed in their natal habitats, i.e., to assess RMR in wild-reared animals. Robert and Bronikowski (2010) reported that at birth, significant differences do not exist in RMR between the two ecotypes, despite differences in immune function, DNA repair rates, and mitochondrial respirometry. Therefore, any differences among adults (after correcting for differences in body size) may not be a traditionally defined genetic effect, but an effect of rearing environment or an epigenetic effect—perhaps different rearing temperatures and/or diets alter gene expression during a lifetime (Bonduriansky and Day 2009). Resting metabolic rate was measured in a closed system (Vleck 1987) for each snake at five temperatures (15, 20, 24, 28, and 32°C) with randomized order of temperature. Rates of oxygen consumption ($\dot{V}_{O_2}$) are expressed in $mL\,h^{-1}$ with volumes corrected to standard temperature and pressure (dry). The specifics of this closed respirometry system are detailed by Robert and Bronikowski (2010). Briefly, animals (equilibrated at test temperature) were sealed in small chambers filled with water-saturated air for a set amount of time (3 h in this study) at a given test temperature, and oxygen concentration within the chamber was measured at the start and at the end of each trial. Chamber temperatures were measured every 30 min with a thermocouple thermometer.

Snakes were randomly assigned to five blocks of 11 individuals each. Within each block, snakes were randomly assigned to a metabolic chamber. The test sequence of temperatures was randomized for each block. This means that some snakes may have proceeded from coldest to hottest temperature, some from hottest to coldest, and all other possible variations. Measurements were made at one temperature each day so that each block required five consecutive days.

Analysis

We performed repeated-measures analysis of covariance to test for the effect of body mass, temperature, and natal ecotype on oxygen consumption (Proc MIXED in program SAS, SAS Institute, Cary, NC, USA). In this model, the natural logarithm (ln) of $\dot{V}_{O_2}$ was the dependent variable. The independent variables included ln(body mass) as a covariate, the within-subjects main effect of temperature and interactive effects of temperature-by-ln(body mass) and temperature-by-ecotype, the latter being the primary effect of interest. Also included in the general linear model was the random effect of population nested within ecotype, and the fixed effect of habitat (tested over the population nested within habitat mean square). The equation for the model was:

$$Y = \mu + T + (T \times M) + (T \times E) + M + E + \varepsilon$$

where T, M, and E are the fixed effects of temperature, mass, and ecotype (and interactions). We tested snakes from three replicate L-fast populations and four replicate M-slow populations.

Results

Effect of body mass: allometry of oxygen consumption

Rate of oxygen consumption increased as a power function of body (Table 2, Fig. 1). This was true at every temperature, and the regression coefficients of $\ln(\dot{V}_{O_2})$ regressed on ln(body mass) were not significantly different ($P = 0.16$, Table 2) across temperatures based on a slope-heterogeneity test (Temperature × ln[mass]) in the analysis of covariance. The pooled coefficient of ln(body mass) in a subsequent simple regression of $\ln(\dot{V}_{O_2})$ on ln(body mass) equaled 0.59, $[\ln \dot{V}_{O_2} = 0.59\ (\pm 0.064) * \ln(\text{mass}) - 1.17\ (\pm 0.19)]$ which suggests that for these snakes, the appropriate scaling factor

Table 2 Repeated-measures analysis of covariance

Source of Variation	df_n, df_d	F	Pr ≤ F
Covariate			
ln(mass)	1, 254	55.7	<0.0001
Between Subjects			
Ecotype	1, 5	18.8	0.0075
Within subjects			
Temperature	4, 254	0.63	0.64
Temperature × ln(mass)	4, 254	1.64	0.16
Temperature × Ecotype	4, 254	3.00	0.02
Error	254		

Dependent variable is ln(rate of oxygen consumption—$mL\,h^{-1}$).

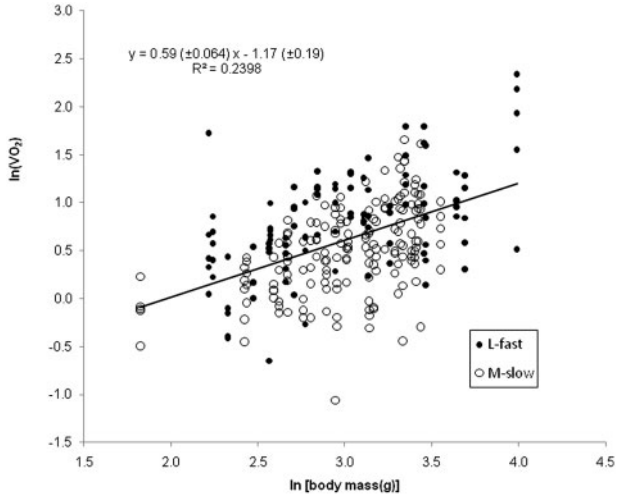

Fig. 1 Regression of $\ln(\dot{V}_{O_2}$ in mL h^{-1}) on ln(body mass in g) for all snakes in the study. The standard errors for the intercept and the coefficient are given in parentheses in the equation for the line.

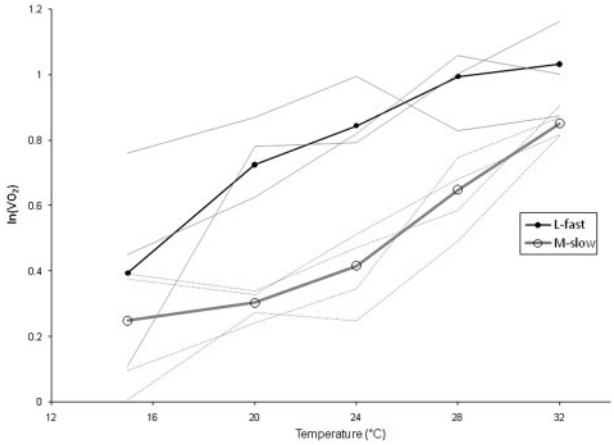

Fig. 2 Least square means from mixed model analysis of covariance of $\ln(\dot{V}_{O_2})$ of fasted snakes measured at five temperatures. The L-fast and M-slow profiles differ significantly at each temperature except 15°C (see text for details). Solid (L-fast populations) and dashed (M-slow populations) grey lines are the least square means for replicate populations nested within ecotypes. Higher means correspond to higher mass-specific metabolic rates, because calculation of these least squares means removed variation associated with body mass (see Model in Materials and Methods).

to describe the allometry of metabolic rate is body mass raised to the 0.59±0.064 power. This value is lower than the range of 0.66–0.80 typically reported for interspecific analyses of metabolic allometry (Speakman 2005b), but well within the range reported for intraspecific analyses in other reptile species (Andrews and Pough 1985; Maxwell et al. 2003).

Effects of temperature and ecotype on rate of oxygen consumption

Temperature (15, 20, 24, 28, and 32°C) and ecotype (L-fast and M-slow) interacted to significantly affect mass-specific oxygen consumption (Table 2). *Post hoc* profile contrasts between the least-square means of neighboring temperatures (Fig. 2) revealed that after accounting for body mass differences, snakes of the M-slow ecotype had significantly lower mass-specific rates of oxygen consumption than did those of the L-fast ecotype at all temperatures excepting 15°C (i.e., M-slow < L-fast at 20, 24, 28, and 32°C). Within ecotype, rates of oxygen consumption at 32 and 28°C were significantly higher than at 15 and 20°C, as expected for ectotherms. For these snakes, 28°C is the optimal—and preferred—body temperature (Peterson et al. 1993); we rarely document adult body temperatures of 32°C or higher except in gravid females.

Discussion

As in other organisms, total oxygen consumption (i.e., metabolic rate) is greater in larger than in smaller snakes, irrespective of ecotype. The manner in which oxygen consumption increases with body size is consistent across temperatures and ecotypes; scaling with body mass raised to the 0.59 power. This value is similar to field metabolic rate scaling reported for a closely related species in the same vicinity (Peterson et al. 1998). Snakes of the L-fast ecotype—with fast growth, early maturation, high reproductive effort, and short life span—reach larger body sizes than snakes of the M-slow ecotype, and consequently, have higher average total metabolic rates (Fig. 1). They also, however, have higher metabolic intensity (mass-specific metabolic rate) than their M-slow conspecifics at any given body size (Fig. 2). Thus, our metabolic intensity results are in agreement with the rate-of-living hypothesis. Higher metabolic intensity in the fast-living ecotype may underlie greater metabolic scope (Calder 1984; Schmidt-Nielsen 1984) and result from selection for faster growth rates, greater size-associated fecundity, foraging and food acquisition rates, and greater travel distances, all observed in L-fast animals.

While the rate-of-living hypothesis suggested that short life spans and long life spans result from differences in metabolic rate, which in principle leads to quicker depletion of energetic resources (Pearl 1928), we do not think the causative links are that direct in this system. For example, at birth, no significant differences exist between L-fast and M-slow RMR measured at 28°C (Robert and Bronikowski 2010). Furthermore, in a comparative study of physiology between long-lived and short-lived species of

colubrid snakes (including a *Thamnophis* species), no significant differences were found in resting metabolic rate (Robert et al. 2007). Instead, we propose that increased oxygen consumption is either one of a suite of physiologically divergent traits between the two ecotypes—whether evolutionarily diverged or due to developmental canalization—or that increased oxygen consumption itself has downstream effects related to oxidative stress (i.e., a causative trait *sensu* the oxidative stress hypothesis).

Higher mass-specific oxygen consumption in the short-lived (and large-bodied) L-fast ecotype has interesting correlates at the cellular level. Mitochondrial isolates from this short-lived L-fast ecotype had lower P:O ratios (ATP produced per atomic oxygen consumed) than those from the M-slow mitochondrial isolates (Robert and Bronikowski 2010). This can be interpreted as less efficient oxidative phosphorylation in L-fast animals, an apparent disadvantage. Alternatively, more uncoupled L-fast mitochondria may actually decrease rates of ROS production, as suggested by Brand (2000), compared to M-slow mitochondria. Free oxygen radicals (ROS) can damage proteins, lipids, and DNA within mitochondria and cells (reviewed for reptiles by Schwartz and Bronikowski 2010). Our earlier work reported higher levels of H_2O_2 in plasma from the short-lived L-fast individuals (Robert and Bronikowski 2010), and higher production rates of H_2O_2 by mitochondria from short-lived species in our interspecific comparison of colubrid snakes (Robert et al. 2007). Postulating that ROS production in general may be evolutionarily divergent between the two ecotypes presupposes that ROS production in mitochondria is a genetically variable trait, or linked to a genetically variable trait, that can respond to natural selection. Very few studies have addressed this question in natural populations, but a recent study (Olsson et al. 2008) documents significant heritability of superoxide production in a lizard species. Whether this is a general pattern is unknown at this time. Other physiological traits that have been measured in these two *Thamnophis* ecotypes demonstrate that erythrocytes of newborn short-lived L-fast animals repair DNA damage less efficiently than do those of newborn, long-lived animals (Robert and Bronikowski 2010). Furthermore, in a more distant physiological axis, innate immune function is heightened in L-fast relative to M-slow snakes (Sparkman and Palacios 2009), consistent with ecoimmunological hypotheses of life history (Lee 2006; Martin et al. 2006). The additional prediction of this hypothesis, that acquired immune function is heightened in M-slow snakes, is currently under investigation.

What mechanism could account for the striking difference between newborn and adult patterns, specifically, that the ecotypes do not differ in metabolic intensity at the newborn stage, but differ consistently, across all regular activity temperatures, at the adult stage? It seems unlikely that we have simply witnessed different degrees of phenotypic plasticity present in each ecotype. Our 1-year common environmental husbandry of the adults suggests that if this were the case, all animals would have acclimated similarly to the laboratory environment. The alternative is that there are permanent differences between individuals of the two ecotypes brought about by either gene expression differences not apparent in newborns, or habitat-specific experiences during development. This could be due to developmental canalization, i.e., that the environment experienced during growth and maturation causes permanent modification of the metabolic trajectory. The selective advantage of such acclimation and fine-tuned metabolic rate over maintaining a large range of phenotypic plasticity could be profound if there are trade-offs associated with plasticity to perform optimally at all temperatures (i.e., *sensu* Huey and Hertz 1984). Furthermore, evolving the ability to acclimate thermally, adjusting to temperature fluctuations, while having overall higher or lower metabolic intensities (L-fast and M-slow, respectively) may select for environmentally-sensitive allelic variants in regulating molecular pathways that also promote longevity (reviewed by Arking and Giroux 2001)

Another—and not mutually exclusive—possibility is that the developmental environment alters the epigenome, which results in permanent changes in gene expression patterns of genes involved in respiration, and potentially ultimately affecting performance and life span. The evolutionary potential of such epigenetic modifications of metabolic function are beyond the scope of this study, but is an exciting new frontier in biology that may be best studied in natural populations that have variation in metabolic rates (reviewed by Bonduriansky and Day 2009). Recent advances in the molecular tool kit of reptile genetics suggest the entirely realistic possibility that molecular network regulation, including epigenetic modification, may soon be studied in natural populations of diverse reptiles.

Funding

US National Science Foundation (DEB 0323379 to A.M.B.).

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