Comparative cellular biogerontology: where do we stand?

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Abstract

Due to the extreme variation in life spans among species, using a comparative approach to address fundamental questions about the aging process has much to offer. For example, maximum life span can vary by as much as several orders of magnitude among taxa. In recent years, using primary cell lines cultured from species with disparate life spans and aging rates has gained considerable momentum as a means to dissect the mechanisms underlying the variation in aging rates among animals. In this review, we reiterate the strengths of comparative cellular biogerontology, as well as provide a survey of the current state of the field. By and large this work sprang from early studies using cell lines derived from long-lived mutant mice. Specifically, they suggested that an enhanced resistance to cellular stress was strongly associated with increased longevity of select laboratory models. Since then, we and others have shown that the degree of stress resistance and species longevity is also correlated among cell lines derived from free-living populations of both mammals and birds, and more recent studies have begun to reveal the biochemical and physiological underpinnings to these differences. The continued study of cultured cell lines from vertebrates with disparate life spans is likely to provide considerable insight toward unifying mechanisms of longevity assurance.

Keywords: reptiles; longevity; fibroblast; stress; senescence

Aging and its associated decline in survival and reproductive fitness is an inherent feature of biological systems (Lemaitre *et al.* 2015). Even bacteria, once thought to possess “clonal immortality,” exhibit signs of replicative aging due to the formation of a distinct “soma” and “germ line” after binary fission (Gomez 2010). Moreover, the mechanisms that drive the aging process, that is the decline of physiological function with the passage of time (Grotewiel *et al.* 2005), are largely conserved from one species to another. Or at least it appears that way. The problem with making this generalization is that the vast majority of biogerontological research has been conducted in one of four species: baker’s yeast (*Saccharomyces cerevisiae*), the nematode worm *Caenorhabditis elegans*, the fruit fly *Drosophila melanogaster*, and house mice *Mus musculus domesticus*. Because these model organisms have been propagated and maintained under the highly artificial conditions of a laboratory setting it is impossible to know whether the regulation of the aging process within these species is a truly a reflection of the aging process under natural conditions or is simply an artifact of laboratory adaptation.

Model Organisms for Biogerontological Research

Laboratory strains of rat, most notably Fischer 344 (F344) and Sprague-Dawley (SD), were the most commonly used rodent aging models up until a few decades ago. Now it is the laboratory mouse, *Mus musculus domesticus* that is undoubtedly the workhorse of vertebrate biogerontological research that is recognized by academics and the general public alike. Unfortunately, a prototypical laboratory mouse does not exist. There are literally hundreds of strains, each with a unique genotype and its associated variation in phenotypic traits, that are used for all aspects of biomedical research (Doetschman 2009). Within biogerontological circles, the C57BL/6 and CB6F1 (i.e. a BALB/c x C57BL/6 F1 hybrid) strains are the mostly widely used. For example, a

Pubmed search using the terms “aging,” “C57BL/6,” and “CB6F1 returned over 750 citations within the last 10 years alone.

The advantages of laboratory mouse strains are several-fold. Most notably: (1) due to standardized husbandry conditions they are relatively easy to maintain with minimal cost; (2) due to their short generation times and large litter sizes it is simple to generate large numbers of individuals for study quickly; and (3) due to their small size hundreds of mice can be kept in a room no larger than a typical bedroom. Most importantly, however, the complete genome of multiple strains has been sequenced and laboratory mice are readily amendable to genetic manipulation (Doetschman 2009). Indeed, genetic manipulation, such as the generation of single, double, and triple gene knockouts, is often used to explore the effects of aging on a broad suite of traits, such as cognition or the DNA damage response (Yu *et al.* 2015, Lemos *et al.* 2010). Finally, since laboratory strains have a mean longevity of approximately two years, it is feasible to complete a life span study within the typical life cycle of a grant (Vanhooren & Libert 2013).

It is also no secret that a given animal model is often chosen for its specific attributes. For example, there are more than 20 strains of mice available from the Jackson Laboratory that harbor mutations associated with both congenital and age-related deafness for those interested in hearing research, as well as dozens more that are susceptible to the development of muscular dystrophies, ocular disease or skin aging (<http://jaxmice.jax.org/research/index.html>). In addition, there is an ever increasing number of single-gene mutant strains that have been shown to be significantly long-lived relative to their wildtype counterparts (Bartke, Sun & Longo 2013). Taken together, this means that laboratory mice have likely become the dominant vertebrate model for aging research more out of convenience rather than any preconceived

appreciation for their utility; although the use of laboratory strains has undoubtedly moved the field of biogerontology forward by enormous leaps within the last two decades.

Nevertheless, it can be argued that this approach is somewhat short-sighted. Just because a model organism is convenient it does not mean it's perfect. Even within mice, there is still much to be learned about what regulates the aging process, especially given the marked disparity among strains in their propensity to develop specific age-related pathologies (Miller & Nadon 2000, Harper 2008). In addition, there is a marked disparity in the lifespan of wild-derived mouse stocks relative to laboratory mice (Harper, Leathers & Austad 2006, Harper *et al.* 2006a). If we go beyond mice and consider mammals as a whole, untangling the aging process becomes even more complex since specific taxa are clear outliers in terms of lifespan potential. For example, bats and primates, especially humans, live far longer than they "should" given their body size (Fischer & Austad 2011, Austad & Fischer 1991, Bronikowski *et al.* 2011).

Another consideration is the influence of laboratory husbandry on a species' genetics and physiology. Regardless of the model organism, all laboratory stocks or strains are adapted to the highly artificial conditions imposed by life in agar plates, plastic vials or shoebox cages under strict nutritional parameters and relatively invariant environmental conditions. Couple this with hundreds-to-thousands of generations of inbreeding for sexually reproducing species, or the continued maintenance of clonal lines for asexually reproducing species, and the conditions are set for very unusual genetic end products. This is a significant deviation from a species' norm and it should come as no surprise that the individual end products are very unlikely to occur in nature. Indeed, some well-known single gene mutations that lead to a dramatic increase in longevity fare poorly

when maintained under “real world” conditions in both invertebrates (Walker *et al.* 2000) and vertebrates (Fabris, Pierpaoli & Sorkin 1972, Giorgio *et al.* 2012). In fact, there is evidence to suggest that free-living populations of typical laboratory-adapted species age differently than their laboratory-adapted counterparts due to the need to contend with the stress of variable environments and social interactions with conspecifics under natural conditions (Authier *et al.* 2012, Bro-Jorgensen 2012, Diamantidis *et al.* 2011, Duyck *et al.* 2010, Hamalainen *et al.* 2014, Hansen *et al.* 2012, Harper *et al.* 2006b, McFarland & Majolo 2013, Reid *et al.* 2010, Reynolds & Phillips 2013, Saino *et al.* 2012, Selman *et al.* 2013, Stumpferl *et al.* 2012, Sutphin & Kaeberlein 2008, Turbill & Ruf 2010, Yuan *et al.* 2013).

Natural Populations of Animals for Aging Research

The variation in maximum lifespans among animals is enormous and Mother Nature has provided a practically limitless array of test species that should garner significant biogerontological interest. For example, maximum life spans may be a 100-fold or more within the same taxonomic class (e.g. mammals, insects) and even within a single mammalian order, namely rodents, differences of at least 30-fold in maximum life span exist among individual species (Hulbert *et al.* 2007). This provides a rich palette from which to work and taking a comparative approach may hold the key to unraveling the complexity of the aging process while identifying the “public” mechanisms of aging long sought after by the biogerontological community (Martin, Austad & Johnson 1996).

In a perfect world, biogerontologists would be able to take a fully comparative approach; i.e. monitoring a diverse array of species with known differences in maximum life spans to catalog age-related changes in gene expression, protein activity and physiological function over chronological time. These data could then be used to search for commonalities among long-lived species, and by extension, the “winning” combination

of genetic and biochemical markers that ensure long life. As beneficial as this scenario would be, it is not a practical reality for two reasons: (1) the infrastructure needed is daunting to say the least, even for an entire institution, much less any one research laboratory. (2) The very reason some species would be desirable, namely the fact that they are exceptionally long-lived, makes them impossible to study simply because no one individual is likely to be around long enough to see the study to its end. On the other hand, due to the relative ease of generating primary cell lines from a diverse array of vertebrate species (Miller *et al.* 2011) it has been possible to delve into the mechanisms underlying successful aging both within, and among, various vertebrate taxa using cell culture models.

Primary Cell Culture, Stress Resistance and Rodent Models of Aging

Life history theory posits that organisms are forced to make physiological trade-offs in order to maximize reproductive fitness (Lemaitre *et al.* 2015). Or more specifically, if a species devotes more resources toward cellular repair and defense, fewer are available for reproduction and *vice versa*. Altogether, it is thought that the product of these tradeoffs is the incurrence of organismal senescence (Williams 1957). Importantly, there is a clear physiological foundation for these tradeoffs and primary cell lines, especially fibroblasts, have been an attractive model system to dissect the specific pathways involved (Jimenez *et al.* 2014a).

Primary cultures of fibroblasts are readily obtainable from any organ that has a large proportion of connective tissue, but skin is the most common source. This is because of the relative ease with which skin samples can be collected using punch biopsies, ear punches and/or toe/tail clips. Moreover, since these methods are minimally invasive and do not require that individuals be sacrificed for biopsy collection they are suitable

for sampling threatened or endangered species. In addition, a single individual could be sampled longitudinally over the course of months or years.

Equally importantly, fibroblasts readily grow under generalized cell culture conditions (i.e. specialized media formulations are not required) and can be derived directly from explants of individual biopsies or after a simple overnight digestion in collagenase. Once established, fibroblasts rapidly proliferate in culture to allow for the generation of large numbers of cells for testing within a short period of time. These methodologies are also generally transferable from one species to the next in that individual cell lines from multiple species can be grown in parallel under identical culture conditions. To date, we have generated primary fibroblast cell lines from more than a thousand individuals representing more than 100 species of mammals, birds, snakes, turtles or lizards. Consequently, we have been able to use primary fibroblast cultures to collect a snapshot of what is happening at the organismal level in species with different aging trajectories (see below). Note that issues concerning data analysis and interpretation, including the impact of species' body mass and phylogeny, as well as the use of specific endpoints generated from cell culture models, has been discussed previously (Miller *et al.* 2011) and will not be covered here.

The utility of this approach first became apparent with the exploitation of significant differences in maximum life span among mutant strains of mice. Most notably, primary dermal fibroblasts that were derived from long-lived Snell, Ames, and growth hormone receptor knockout (GHRKO) dwarf mice were generally more resistant to the lethal effects of a diverse array of cytotoxic agents relative to cells derived from their normal-lived, non-mutant littermates (Murakami, Salmon & Miller 2003, Salmon *et al.* 2005, Salmon, Ljungman & Miller 2008). In addition, it was shown that cell lines from Snell dwarf mice are better able to resist the inhibitory effects of rotenone and low glucose

media on cellular physiology (Leiser, Salmon & Miller 2006, Leiser & Miller 2009), were better able to repair UV-induced DNA damage (Salmon, Ljungman & Miller 2008) and can resist the potentially toxic effects of long-term culture under ambient (i.e. 20%) oxygen (Maynard & Miller 2006). Intact dwarf mice are also more resistant to the toxic effects of an array of pharmacological stressors (Bokov *et al.* 2009) while exhibiting altered patterns of mitogen activated protein kinase (MAPK) signaling in the liver (Sun *et al.* 2011). Although the underlying mechanism for these differences in cellular stress resistance remain unclear, a direct effect of growth hormone signaling on the ontogeny of cellular physiology (Panici *et al.* 2010) has been suggested and may involve alterations in mammalian target of rapamycin (mTOR) signaling (Li & Miller 2015) and/or autophagic (Wang & Miller 2012a, Wang & Miller 2012b) pathways.

Interestingly, cells grown from dietary models of life extension fail to show this correlation. More specifically, dermal fibroblasts from mice subjected to life-long caloric restriction (CR) or provided with a diet low in the essential amino acid methionine, were no more stress resistant to multiple cytotoxins relative to their normal-fed counterparts (Harper *et al.* 2006c). Caloric restriction is perhaps the most robust life-extending intervention known (Fontana & Partridge 2015) while diets low in methionine have been repeatedly shown to increase longevity in both rats and mice (Perrone *et al.* 2013, Sun *et al.* 2009, Miller *et al.* 2005). A clue to this apparent discrepancy comes from studies using conditioned media; or more specifically, cells exposed to media supplemented with serum collected from rodents undergoing CR are more stress resistant than are cells grown in the presence of normal media alone. This suggests the presence of specific circulating factors needed for the life extending effects of dietary restriction that are lost during the derivation and expansion of individual cell lines (de Cabo *et al.* 2015, de Cabo *et al.* 2003).

Nevertheless, there have been several studies using other genetic mouse models of longevity that have also failed to show a significant difference in stress resistance among long-lived versus control littermates. This includes cells grown from mice globally deficient in insulin receptor substrate-1 (Page *et al.* 2014), as well as whole animal studies using mice with a brain-specific deletion of the insulin-like growth factor-1 receptor (Kappeler *et al.* 2008). Moreover, in contrast to the findings typical of Snell dwarf mice, pharmacological induction of endoplasmic reticulum (ER)-stress using both tunicamycin and thapsigargin indicated that Snell dwarf mouse fibroblasts were significantly more sensitive to ER stress than their littermate controls (Sadighi Akha *et al.* 2011). Although it's still not clear what may account for these observations, differential regulation of the somatotrophic axis has been suggested (Page *et al.* 2014, Kappeler *et al.* 2008, Sadighi Akha *et al.* 2011) which may be confounded by the use of a specific genetic background (Mulvey, Sinclair & Selman 2014).

Primary Cell Culture and Stress Resistance in Vertebrate Dermal Fibroblasts

Consistent with the results obtained using cells from long-lived mutant mice, the stress resistance profile of fibroblasts grown from a variety of mammalian taxa has also indicated that there is a correlation between the degree of stress resistance and species life span. In particular, Kapahi *et al.* (Kapahi, Boulton & Kirkwood 1999) first showed that the resistance of fibroblasts to oxidative stressors was positively correlated with mammalian longevity across several taxonomic orders. More recently, our own work has built upon this finding and has shown that primary skin-derived fibroblasts from young adult mammals of multiple species (8 rodents and 1 bat) also exhibit a clear relationship between species maximum life span and the degree of stress resistance to multiple classes of stressor even after controlling for the potentially confounding effects of body size and phylogeny (Harper *et al.* 2007). Cells from the exceptionally long-lived Little Brown Bat tended to be the most resistant to each of these stressors, while cells

grown from wild-caught house mice were more stress resistant than those from a genetically heterogeneous laboratory stock (Harper *et al.* 2007). Indeed, the latter finding is consistent with whole animal studies of wild-derived versus laboratory adapted stocks of mice (Miller *et al.* 2002, Harper *et al.* 2006a, Hulbert *et al.* 2006), as well as the stress resistance profiles of cell lines grown from other strains of wild-derived mice (Yuan *et al.* 2006). Finally, the resistance of dermal fibroblasts grown from naked mole rat, the longest-lived rodent (Edrey *et al.* 2011), was generally in agreement with the notion that an enhanced ability to resist the physiological effects of a diverse array of stressors goes hand-in-hand with increased longevity among species (Salmon *et al.* 2008). However, it should be noted that naked mole rat fibroblasts were especially sensitive to ER stressors, hydrogen peroxide and UV-C radiation which may reflect the evolutionary adaptations associated with a subterranean lifestyle (Salmon *et al.* 2008).

Complementary studies using cultured bird cells also supports the idea that stress resistance and longevity go hand-in-hand. For example, an early study of oxidation resistance using cultured renal cells from 3 bird species (i.e. budgerigars, starlings, canaries) showed that cells from each were more resistant to oxidative stress and DNA damaging agents than was a mouse cell line tested in parallel (Ogburn *et al.* 1998). A second study extended these findings to include stress resistance patterns within birds by comparing cells grown from short-lived Japanese quail to those grown from longer-lived budgerigars (Ogburn *et al.* 2001). Finally, we recently completed a more inclusive survey using cell lines derived from 35 bird species from 8 different Orders and also found that the degree of stress resistance among species was significantly correlated with species maximum life span (Harper *et al.* 2011). We also confirmed that, as a whole, avian cells are significantly more resistant to stress than are mammalian cells derived from same-sized individuals (Harper *et al.* 2011).

In addition to the extreme variation in organismal physiology seen among species, life-history theory also postulates that individual populations within species will lie along a “slow-fast” life-history axis in accordance with the demands imposed by their ecological space (Saether 1988, Saether & Engen 2015). Ultimately, this axis is characterized by a low reproductive output in conjunction with a low extrinsic mortality rate at the whole animal level in those species lying at the “slow” end of the continuum. By extension species that lie at the “fast” end of the continuum have relatively high rates of reproduction and extrinsic mortality. Ultimately, these tradeoffs will reflect the underlying genetic architecture of the individual whose structural detail is retained at the cellular level (Mitrecic *et al.* 2008).

As recently reviewed by Jimenez *et al.* (Jimenez *et al.* 2014a), north temperate versus neotropical bird species are an excellent example of the “slow-fast” model system. In particular, tropical birds tend to grow more slowly and have reduced whole-animal basal metabolic (BMR) and peak metabolic (PMR) rates, as well as a reduction in the size of metabolically active organs, compared with temperate birds. Tropical bird species also tend to have higher survival rates. Using this model system, we showed that cell lines from “fast-living” temperate birds were significantly less resistant to stress than were cell lines derived from phylogenetically matched “slow-living” species from the tropics (Jimenez *et al.* 2013). Cellular metabolic rates were also higher in dermal fibroblasts from “fast-living” temperate species relative to cells grown from tropical species using basal, uncoupled, and non-mitochondrial cellular O₂ consumption (OCR), proton leak, and anaerobic glycolysis (extracellular acidification rates [ECAR]) as indicators (Jimenez *et al.* 2014b).

Can Primary Cell Lines Provide Clues to Anti-Aging Mechanisms?

Despite the general association between cellular stress resistance and species life span in both mammals and birds, only recently have the biochemical and physiological underpinnings to these differences begun to reveal themselves. For example, both genetic and pharmacological models of life span extension in mice showed that proteasome activity is maintained at a higher level with age relative to shorter-lived controls (Chondrogianni *et al.* 2014). This is true of the proteasome activity of naked mole rat organs in that it is significantly greater with age relative to mice (Rodriguez *et al.* 2012, Rodriguez *et al.* 2014, Azpurua *et al.* 2013). Moreover, studies using primary dermal fibroblasts from mammals with disparate life spans have supported this notion (Pickering *et al.* 2014, Pickering, Lehr & Miller 2015, Pride *et al.* 2015) as well. This isn't surprising given that the ability to cope with both genotoxic and oxidative stressor is at least partly dependent on a functional proteasome (Ermolaeva, Dakhovnik & Schumacher 2015, Aiken *et al.* 2011).

That being said, significant differences in other aspects of cellular physiology amid short- versus long-lived species of vertebrate have also been observed. For example, significant differences in metal ion dynamics among species (Dostal *et al.* 2014), and variation in the specific composition of mitochondrial membrane lipids (Calhoon *et al.* 2014), has also been observed among short versus long-lived species. In addition, the kinetics of the mitogen activated protein kinase (MAPK) response, or more specifically variation in the induction times of the extracellular signal related kinase (ERK) 1/2 effector molecule (Elbourkadi, Austad & Miller 2014) differs among short- versus long-lived species, as well as the expression of nuclear factor erythroid 2-related factor (Nrf2) dependent genes (Lewis *et al.* 2015). Indeed, cells from naked mole rats have significantly higher constitutive levels of Nrf2 protein, as well as the tumor suppressor p53, in conjunction with halted cell proliferation in the face of cytotoxin challenge (Lewis *et al.* 2012).

Finally, studies of telomere biology within mammals suggests that the evolution of homeothermy in conjunction with altered telomere dynamics may have contributed to differential stress resistance within, and among, species (Gomes *et al.* 2011). This may be due, at least in part, to its effect on cellular growth rates in conjunction with differential telomerase activity among rodents. For example, Seluanov *et al.* (Seluanov *et al.* 2008) found that species with a large body mass lack telomerase activity and have cells that undergo replicative senescence in conjunction with their increased lifespans. On the other hand, cells grown from small, short-lived rodents were found to maintain telomerase activity, and did not undergo replicative senescence while showing rapid growth. Cells from small, long-lived rodents were in that middle in that they possessed telomerase activity but tended to grow slowly in culture. There was no relationship between the occurrence of cellular senescence and species lifespan, however, although body mass and senescence were correlated. Altogether increased longevity in conjunction with reduced cellular growth rates appears to have evolved multiple times within rodents but whether this applies to other mammalian lineages remains unknown.

Stress Resistance Profiles of Cell Lines from Non-Avian Reptiles

Reptiles are the sister clade to mammals, and together with mammals comprise the Amniotes (Hedges 2012). Moreover, the reptiles can be roughly broken into two groups: (1) snakes, lizards, crocodilians and turtles (i.e. non-avian reptiles); and (2) birds (i.e. avian reptiles). Notwithstanding, non-avian reptiles in particular have largely gone ignored by the aging research community despite there being little evidence to suggest that they suffer from the senescent decline seen with mammals and birds (Nussey *et al.* 2013). In fact, the oldest individuals in natural populations of non-avian reptiles are often the most fecund and physiologically robust (Miller *et al.* 2014, Congdon *et al.* 2001, Congdon *et al.* 2003, Henry 2003, Sparkman, Arnold &

Bronikowski 2007, Miller 2001). Undoubtedly, the prodigious stress resistance exhibited by some species, such as anoxia tolerance in otherwise aerobic species of turtles, contributes to their extreme longevities (Krivoruchko & Storey 2010).

It is often difficult to pinpoint the most likely cause of death in all but the best-known vertebrate models. Nevertheless, the occurrence of specific classes of lesions, such as neoplastic disease and neurodegenerative disorders, increases almost universally among animals with increasing age (Gems & Partridge 2013). This may not be true for non-avian reptiles, however. For example, a comprehensive review of marine turtle necropsies indicated that a wide range of pathological lesions occur more commonly with increasing in age, but at a relatively low frequency (Flint *et al.* 2009). Moreover, these lesions were deemed unlikely to have contributed directly to an individual's death; instead most were thought to be the consequence of human-caused alterations to the environment and not an intrinsic biological process *per se* (Flint *et al.* 2009). Likewise, despite respiratory tract disease being a common cause of morbidity and mortality within captive reptiles, it is most often a consequence of improper environmental housing conditions and not an underlying physiological process (Schumacher 2003).

In addition to pathology, whole animal studies using non-avian reptiles are consistent with the maintenance of physiological competence with aging in this group. For example, measures of immune function in both snakes (Palacios, Sparkman & Bronikowski 2011) and turtles (Zimmerman *et al.* 2013, Schwanz *et al.* 2011) show no evidence for an appreciable decline with advancing age. A retrospective study of captive swamp turtles in Australia also showed that older individuals were more resistant to an opportunistic dermal infection than were young individuals (Ladyman *et al.* 1998). The same appears to be true of changes in antioxidant (Mishra & Patnaik 1993) and glycolytic activity (Jena, Mohanty & Patnaik 1991), as well as endocrine

measures (MahapatraMahata & Maiti 1987), within aged populations. Finally, aging is not associated with a significant decline in telomere length in leatherback turtles (Plot *et al.* 2012) and pythons (Ujvari & Madsen 2009); although this may not be so in a second snake species (Bronikowski 2008). However, given that the relevance of telomere length to organismal aging is still unclear the significance of these data is unknown (Koliada, Krasnenkov & Vaiserman 2015).

Indeed, because turtles are so longevous, a recent evaluation of conservation strategies has determined that relatively few individual females are needed to maintain viable numbers within otherwise fragmented and threatened populations (Shoemaker *et al.* 2013). This is because the likelihood that any given female turtle will survive for decades while maintaining a high level of fecundity is relatively high. Ultimately, this results in an effectively large breeding population despite the small individual numbers of females. This is in opposition to the typical conservation strategy that employs a minimum viable population whereby individual females are at a premium. This finding also highlights the significance the unique aging process that seems to be inherit to many non-avian reptiles.

Because they are sister clades, mammals and reptiles exhibit a large degree of homology with regard to their anatomy, physiology, genome structure and gene function. Indeed, the relationship between aging and specific life history traits, such as body mass and age at maturity, are conserved among all amniotic vertebrates (Ricklefs 2010). Genomic profiling of multiple non-avian reptiles also supports the notion that specific genetic networks underlying physiological function are common to all vertebrates (Castoe *et al.* 2013, Shaffer *et al.* 2013, McGaugh *et al.* 2015). What remains unknown, however, is the mechanism by which non-avian reptiles apparently

slow the rate of senescence decline typically seen within endothermic vertebrates (Nussey *et al.* 2013).

The ability of dermal fibroblasts to resist the lethal effects of cytotoxic agents is correlated with species longevity among both mammals and birds (Harper *et al.* 2007, Harper *et al.* 2011, Jimenez *et al.* 2013, Salmon *et al.* 2008), as well as strains of mice harboring genetic mutations that result in a significant lifespan extension (Murakami, Salmon & Miller 2003, Salmon *et al.* 2005, Leiser, Salmon & Miller 2006, Salmon, Ljungman & Miller 2008, Sadighi Akha *et al.* 2011, Panici *et al.* 2010). In accordance with these studies, we recently evaluated the stress resistance properties of snake dermal fibroblasts exposed to graded doses of cytotoxic and metabolic stressors. More specifically, we generated three unique cell lines: two independent garter snake (*Thamnophis elegans*) cell lines and a single cell line derived from a second snake species, namely the corn snake (*Pantherophis guttatus*). In each case, the garter snake cell lines were grown from tail biopsies collected from multiple wild-caught females that had been maintained in captivity for up to one year prior to sampling. Each cell line, referred to as *T. elegans* Line 1 and *T. elegans* Line 2, was generated by pooling early passage cells from multiple individuals and maintaining these mixed, heterogeneous cultures as unique “individuals” over time; the specific individuals that contributed to each pool did not overlap. The corn snake line was derived from a single female obtained from a commercial breeder. The specific age of the individual donors was unknown regardless of species. The results of this preliminary study are detailed in Table 1.

Two things stand out from these data. First, within the garter snake there is a marked degree of variation in the ability to withstand lethal and metabolic stress among the individual cell lines; that is, regardless of the stressor used one cell line (*T. elegans* Line

2, second column Table 1) tended to be more resistant to a given stressor than are cells from the other line. Second, consistent with the data for endothermic reptilian cells (i.e. birds), we find that snake cells appear to be more resistant to stress than were their mammalian counterparts. In particular, the LD₅₀ (amount of cytotoxin needed to kill 50% of the population) of a population of snake fibroblasts is several times greater than the LD₅₀ of a population of rodent cells; snake fibroblasts are also more resistant to glucose deprivation than are fibroblasts from these same species (see Harper *et al.* 2007 for details).

To delve deeper into the stress resistance properties of snake cells, we generated lines for a set of 30 individual garter snakes that were distributed across the adult lifespan (ca. 4 – 12 years of age), and that represented two ecotypes of naturally occurring garter snake. Akin to the work done with birds that lie along the “slow-to-fast” pace of life axis, extensive work done with populations of garter snakes from the area around Eagle Lake in the Sierra Nevada Mountains in California has revealed the existence of “slow-aging” (SA) and “fast-aging” (FA) ecotypes of garter snakes in these populations. Whereas for the preliminary studies in the above paragraphs, we used several animals of both SA and FA ecotypes to represent a “garter snake,” here we developed individual-level dermal fibroblast lines to compare cell stress physiology between SA and FA snakes. The characteristics used to define these ecotypes have been covered extensively elsewhere (Bronikowski 2008, Bronikowski & Vleck 2010), but, in brief, whole animal studies have revealed significant genetic and physiological differences among ecotypes that are consistent with the predictions of life history theory. For example, SA garter snakes have more efficient hepatic mitochondria, as well as more efficient DNA repair capacity, relative to FA garter snakes (Robert & Bronikowski 2010) and each ecotype exhibits a coordinated response in a suite of metabolic parameters that are linked to maximum lifespan (Robert, Brunet-Rossinni & Bronikowski 2007).

Significant variation at candidate nodes within the genetic and biochemical network that comprises the physiological response to stress has also been found (Schwartz & Bronikowski 2013). Interestingly, others have found that these findings are mirrored in slow versus fast-aging populations of free-living lizards (Lucas & French 2012).

As indicates above, we used tail snips to establish more than 70 individual cell lines from an approximately equal number of females collected from distinct populations within in ecotype. In each case, roughly one-half of the females had been pregnant at the time of capture. Using our established protocols (Jimenez *et al.* 2013, Harper *et al.* 2011, Harper *et al.* 2007) we tested the ability of individual cell lines from each ecotype to resist the lethal effects of methyl methanesulfonate (MMS; a DNA-alkylating damaging agent) and paraquat (an oxidizing agent), as well as the metabolic stress imposed by glucose limiting culture conditions. *A priori*, the hypothesis was that cells grown from individuals of the SA ecotype would be more stress resistant than cells grown from FA snakes, and although we did find that cells from SA snakes tended to be more resistant to the lethal effects of multiple cytotoxins, statistically significant findings tended to depend on a female's body size, ecotype and sometimes reproductive status (Table 2, p-values not adjusted for 1-tailed ecotype prediction). It's important to note that garter snakes exhibit indeterminate growth over their adult lifespans and we have shown previously that size and age are highly correlated (Robert, Brunet-Rossinni & Bronikowski 2007, Sparkman, Arnold & Bronikowski 2007); thus larger animals are older.

From this preliminary analysis, we find that cell lines grown from larger/older females were more resistant to the lethal effects of MMS than were cell lines grown from smaller/younger females irrespective of ecotype if the donor female was non-pregnant (Figure 1A). On the other hand, larger/older females of the FA ecotype, but not the SA

ecotype, were less resistant to MMS cytotoxicity if the donor female was pregnant. These findings suggest that there is a gestational cost that acts at the level of the cell, and that this cost is manifest in FA, but not SA females. In the case of metabolic stress imposed by glucose limitation, there is no indication that there is gestational cost; instead we found that for SA females, cell lines from younger donors were more resistant to glucose limitation (lower residuals in Figure 1B), whereas this age-effect was not seen in FA animals.

It is also noteworthy that the estimated stress resistance parameters for each ecotype were in line with our preliminary studies (Table 1). This lends further support to the conclusion that cell lines from non-avian reptiles may exhibit extreme cellular stress resistance in comparison to mammals. Nevertheless, due to the large number of individual cell lines, it was impossible to test each line in parallel. Consequently, individual cell lines were tested in three discrete “batches” using approximately 24 individual cell lines over the course of three consecutive weeks. This is important because we found marked variation among individual test batches in the estimated degree of stress resistance for each of the three stressors tested regardless of ecotype. Undoubtedly, this variation significantly weakened the power of our statistical models and suggests that future studies will need to account for inter-assay variability. For example, an equal number of individual cell lines grown from donors of the same sex, age and/or reproductive status among ecotypes should be tested in parallel within a given batch. Likewise, inter-specific comparisons should strive to test an equal number of cell lines per species within a given batch. Future studies that use cell lines from donor snakes of both sexes, of the same relative age and of the same reproductive status at the time of capture are needed. The assessment of stress resistance parameters from other species of non-avian reptiles such as turtles, lizards and crocodilians is also needed.

Concluding Remarks & Future Directions

The notion that increased stress resistance would go hand-in-hand with increased longevity is intuitive and gained strong initial support from both whole animal and cell-based studies using model organisms. Moreover, these initial findings have been extended to include cells lines “from the wild,” in that cells grown from longer-lived individuals of natural populations tend to be more stress resistant than those from shorter-lived individuals. Moreover, since this finding has been shown repeatedly among vertebrate cell lines, it is especially compelling and suggests that taking a comparative approach using measures of cellular function may be the key to finding public mechanisms of aging. Although, it remains to be seen whether cellular stress resistance and longevity correlates within additional vertebrate taxa such as fishes and amphibians. In addition, the incorporation of phenotypes beyond cytotoxin resistance, such as the induction of a cellular inflammatory response (Finch *et al.* 2010), the ability to withstand osmotic stress and/or the generation of metabolomic profiles, are lacking and are of interest.

Moreover, comprehensive data for putative stress regulatory mechanisms among species with differential aging trajectories are regrettably absent across most phylogenetic boundaries. That is, studies using multiple individuals from higher-order taxonomic groupings, such as all mammals or all amniotic vertebrates, are lacking. This is important because it’s difficult to infer whether or not a putative anti-aging mechanism that has been identified within a given cell line is indeed a public mechanism of aging or is simply a reflection of individual aging trajectories within a given phylogenetic lineage. Unbiased studies, such as siRNA screens for modulators of cellular stress resistance across multiple species, are sorely needed and would identify candidate pathways. This could be followed by the manipulation of these candidate pathways among species

to determine whether the effect on stress resistance is conserved. Finally, if clear “winners” are identified, this work could be moved into model organisms to examine the effect on individual longevity.

Finally, it remains to be seen whether the observed differences among dermal fibroblasts grown from individuals with disparate longevity translates to other cell types. It’s possible the correlation between the cytotoxic stress resistance of dermal fibroblasts and species longevity is simply a quirk, especially given that dermal fibroblasts are not likely to contribute directly to the aging process in intact animals. Yet, the repeated demonstration of this relationship among multiple vertebrate lineages argues against this notion. Perhaps the relatively newfound ability to induce dermal fibroblasts to become pluripotent stem cells (i.e. iPSC), from which all other cell types can be derived, will allow the gap between dermal fibroblasts and cell types of greater relevance to biogerontologists to be bridged.

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Table 1. Stress resistance of primary dermal fibroblasts from two snake species. Values are the LD₅₀ or 50% inhibitory dose for cytotoxins and glucose limiting conditions, respectively.

Stressor*	Cell Line		
	<i>T. elegans</i> Line 1	<i>T. elegans</i> Line 2	<i>P. guttatus</i>
Cadmium	189.0 ± 2.8	416.1 ± 6.2	216.8
Paraquat	49.6 ± 7.7	54.7 ± 1.4	57.0
Hydrogen Peroxide	1155 ± 47	1619 ± 77	598
MMS	2.0 ± 0.07	2.1 ± 0.04	1.8
Tunicamycin	34.5 ± 1.1	39.1 ± 1.7	41.9
KCN	3.6 ± 0.2	5.6 ± 0.5	3.1
Glucose	0.026 ± 0.002	0.042 ± 0.003	0.046

* Values for each garter snake cell line (*T. elegans*) is the mean ± SEM of individual cell lines tested in parallel across multiple assays (n = 12); for the corn snake (*P. guttatus*) values are the estimated value from a single assay. Units for LD₅₀ values are in μM (cadmium, H₂O₂), mM (MMS, KCN and paraquat), mg/mL (glucose) or μg/mL (tunicamycin).

Table 2. Stress resistance of fast-aging (FA) and slow-aging (SA) garter snake primary dermal fibroblasts. Dependent variables are either LD₅₀ (Paraquat and MMS) or ID₅₀(50% inhibitory dose for glucose limiting conditions). The general linear model for each dependent variable (paraquat, MMS, and glucose) contained the random effect of Population-nested-within-Ecotype, as well as the covariate of body length, which is a proxy for age in these populations. Paraquat data were log-transformed for normality; MMS and Glucose data met normality assumptions. We predicted that the SA ecotype would demonstrate overall greater resistance, alone and in combination with over variables. P-values have not been adjusted accordingly for this 1-tailed test. But for ecotype main and interaction effects, we consider P <0.10 to be marginally significant.

	Log Paraquat ¹		MMS		Glucose	
Source of Variation	F _{df1,df2}	Pr >F	F _{df1,df2}	Pr >F	F _{df1,df2}	Pr >F
Batch	12.78 _{2,44}	<0.0001	52.9 _{2,48}	<0.0001	5.41 _{2,48}	0.0076
Ecotype	1.57 _{1,5}	0.27	2.13 _{1,5}	0.20	2.79 _{1,5}	0.16
Pregnant	0.26 _{1,44}	0.61	9.77 _{1,48}	0.003	2.36 _{1,48}	0.13
Eco x pregnant	0.02 _{1,44}	0.89	4.26 _{1,48}	0.04	0.77 _{1,48}	0.38
Body Length	0.48 _{1,44}	0.49	0.01 _{1,48}	0.91	1.88 _{1,48}	0.18
Length x Eco	1.48 _{1,44}	0.23	2.17 _{1,48}	0.14	3.72 _{1,48}	0.06
Length x Pregnant	0.17 _{1,44}	0.68	8.77 _{1,48}	0.0048	2.62 _{1,48}	0.11
Length x Pregnant x Eco	0.19 _{1,44}	0.67	2.95 _{1,48}	0.09	1.18 _{1,48}	0.28

Legends to figures

Figure 1. Significant interactions that involved Ecotype (i.e., Slow-aging versus Fast-aging) for: A) MMS LD₅₀ (Ecotype x Pregnant-Status x Length) and B) Glucose ID₅₀ Ecotype x Length. Values for each panel are residuals from the full model without body-length plotted against body-length to visualize the directionality of the effect of body length. A) Larger/older FA ecotype pregnant females are less resistant to the DNA damaging MMS reagent than are younger pregnant FA females and all SA females irrespective of whether they are pregnant or not. For non-pregnant females, older/larger individuals are more resistant to MMS as indicated by the greater LD₅₀ residuals. B) Cell lines from smaller/younger SA females were more resistant to glucose depletion than were those from larger/older SA females; cells from FA females were similarly resistant to glucose limitation regardless of size/age.

Figure 1

A) MMS residuals versus Body Length

B) Glucose residuals versus Body Length