The free-radical damage theory: Accumulating evidence against a simple link of oxidative stress to ageing and lifespan

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Recent work on a small European cave salamander (*Proteus anguinus*) has revealed that it has exceptional longevity, yet it appears to have unexceptional defences against oxidative damage. This paper comes at the end of a string of other studies that are calling into question the free-radical damage theory of ageing. This theory rose to prominence in the 1990s as the dominant theory for why we age and die. Despite substantial correlative evidence to support it, studies in the last five years have raised doubts over its importance. In particular, these include studies of mice with the major antioxidant genes knocked out (both singly and in combination), which show the expected elevation in oxidative damage but no impact on lifespan. Combined, these findings raise fundamental questions over whether the free-radical damage theory remains useful for understanding the ageing process, and variation in lifespan and life histories.

Keywords:
- ageing; free radicals; life histories; lifespan; oxidative stress

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Abbreviations:
DR, dietary restriction; **ROL,** rate of living; **SOD,** superoxide dismutase; **SPF,** specific pathogen-free.

Deep within aquifer caves in the Dinaric karst in the Balkan countries there lives a small aquatic salamander (*Proteus anguinus*). It is an innocuous, seldom observed animal, about 25 cm long when adult, that is classically adapted to its cave environment – it is blind, its skin is unpigmented and it is incredibly tolerant both to high levels of anoxia and food deprivation [1]. The 'olm' or 'human fish' as it is commonly called lives a life almost free from predation. In line with the evolutionary theory of ageing [2], which suggests that extremely low extrinsic mortality risks should drive the evolution of exceptional longevity, it lives a long time, indeed remarkably long for its size. Zoo specimens have been reported to live up to 70 years of age. However, a recent study of the olm, based on weekly mark-recapture statistics of a population that was established in a cave in southern France in 1952, has revealed that its annual survival is 0.984. This study has led to the prediction that olms live on average 68.5 years, and have a predicted maximum lifespan of over 100 years [3]. These data suggest that the lifespans reported for captive animals were far from exceptional. Indeed the 'olm' has by far the longest lifespan of any known amphibian. Perhaps even more noteworthy, however, is that additional studies of the physiology of this animal [1, 3] have added to a growing body of work calling into question one of the most popular ideas explaining the physiological basis of ageing and life history trade-offs – the free-radical/oxidative damage theory.

The free-radical theory of ageing has its origins in the early 1950s when there was intense research interest in the damaging effects of ionising radiation, such as was experienced in the aftermath of the atomic weapons used on Hiroshima and Nagasaki at the end of World War II. Around this time, radiation chemists and biologists realised that the horrendous damage induced by ionising radiation was caused primarily by free-radical damage to macromolecules such as DNA, lipids and proteins. Subsequently the paradigm shifting idea emerged that commonality existed between the macromolecular damage caused by radiation and macromolecular damage

associated with ageing based on the same free-radical source. These observations were highly significant given that free radicals are a spontaneous and natural by-product of aerobic metabolism, formed principally during oxidative phosphorylation. In the course of this process, electrons derived from the citric acid cycle pass along the cytochrome proteins on the inner mitochondrial membrane and react promiscuously with free oxygen, leading to the production of an oxygen radical (O^{-}). This occurs in both complex 1 and complex 3 of the electron transport chain [4], and estimates of the amount of consumed oxygen that ends up as oxygen radicals vary from 0.3 to 3%. Oxygen radicals may react with other compounds to form a variety of additional radicals (more specifically reactive oxygen/nitrogen species) all of which are capable of causing cellular damage. Although the figures for oxygen radical production as a percentage of oxygen consumption are probably too high, the potential damaging effects of free radicals have led to the evolution of a plethora of protective mechanisms that aim to detoxify the radicals before they cause damage, or to repair or mitigate this damage. These have been reviewed in detail elsewhere [5] but include the antioxidant superoxide dismutase (SOD) that converts superoxide (O_2^{-}) to hydrogen peroxide (H_2O_2), glutathione peroxidase and catalase that degrade H_2O_2 to water and oxygen, the base excision, nucleotide excision and double-strand break DNA repair systems, thiol-containing molecules, phase-2 detoxifying enzymes and the proteosomal system. However, effective as these defence systems may be, some radicals always evade the protection system and some of the resultant damage always remains unrepaired. As a consequence damage appears to slowly accumulate with age [6] until it starts to compromise the function of the organism as a whole [5]. The idea therefore soon took hold that the reason we age and ultimately die is because of the accumulated damage that these oxygen free radicals and their derivatives cause [7]. This idea was immediately attractive because it provided a physiological mechanism for what was at the time the dominant theory of ageing – the 'rate of living theory' (ROL).

The ROL theory has its origins at the start of the last century when it was observed that larger species normally live longer than smaller ones and they also have lower metabolic rates (oxygen consumption per gram of tissue). Critically it was suggested that across species the product of the lifespan and mass-specific oxygen consumption rates was constant. The tissues of different species therefore seemed to burn through a fixed amount of energy, and hence oxygen consumption, before the organism expired. Some animals like mice burned through the energy fast and lived short lives, while others burned through it more slowly and lived longer. Living fast (consuming oxygen at a high rate) generally means dying young. The free-radical theory, where oxygen radicals are generated in direct proportion to oxygen consumption and damages tissues causing them to lose functionality, therefore provided a cogent explanation for these data.

By the late 1970s the ROL theory had fallen out of favour, principally because as more data accumulated it was demonstrated that particular groups of animals did not follow the 'living fast dying young' pattern. Birds, for example, had very high rates of metabolism when at rest and when actively engaged in flight, the most costly form of physical activity [8], yet lived substantially longer than equivalent sized mammals [9, 10]. Among the mammals, bats showed similar anomalously long lives [11], yet marsupials with low metabolic rates seemed, if anything, to live shorter lives than eutherian mammals [12]. Clearly there was no fixed link between energy metabolism and ageing (reviewed by Speakman [13]). While the ROL theory fell into disrepute, the free-radical theory of ageing gained in popularity, in part because it was felt that the free-radical theory could explain these anomalies and triumph where the ROL theory had failed. Hence, it was shown that bird mitochondria produce very much lower levels of free radicals, for a fixed level of oxygen consumption [14, 15]. These observations were matched by many studies that showed correlations across species in parameters related to oxidative damage and maximal lifespan of the species in question [16, 17].

Within species, a considerable body of data exists indicating that, at least at the correlative level, ageing is accompanied with an increase in oxidative stress [18–21]. These data were accompanied by observations that dietary restriction (DR), the only environmental manipulation known to extend both mean and maximal lifespan [22], was associated with a decline in the level of age-related oxidative damage [23–27]. Moreover, links were shown between oxidative damage and the rate of telomere shortening [28], with extracellular SOD also slowing telomere shortening in vitro [29]. From the late 1990s until the mid-2000s the free-radical theory of ageing was the dominant mechanistic idea as to why we age and die.

Ecologists were relatively slow to catch on to the free-radical bandwagon (e.g. [30]). However, it became apparent that the free-radical theory potentially provides a more general framework for understanding not only ageing but life history trade-offs. Life history theory is the study of how organisms combine defining aspects of their lives such as their fecundity, development and lifespan and how such combinations have evolved. The dominant paradigm in the field is the idea that animals cannot simultaneously maximise all their history traits. Hence life history traits must trade-off against each other [31]. The mechanisms underpinning these trade-offs remain unclear, but this area has long had at its core the idea that energy utilisation is an important part of the picture [32]. The assumption that free radicals are produced in proportion to energy utilisation provided ecologists with a suitable mechanistic framework to understand life history trade-offs (e.g. [30, 33]), and several reviews to this effect have appeared recently [34–36]. However, by the time ecologists were embracing the free-radical theory, cracks in the free-radical theory were already starting to show amongst gerontologists concerned with fundamental mechanisms of ageing [37–39].

Perhaps the first clues to the problems with the free-radical hypothesis started in the late 1990s with improvements in our understanding of mitochondrial biology. As our knowledge in this area began to expand it became clear that the simple 1:1 link between energy metabolism and free-radical production rates was far too simple, and that increased oxygen consumption does not inevitably increase mitochondrial radical production [40]. In addition, oxygen radical production by mitochondria might also be critically affected by the mitochondrial inner membrane potential [41]. This is because the inner membrane potential has a major impact on the

likelihood of a promiscuous reaction occurring between oxygen and the electrons passing along the electron transport chain. This led to the paradoxical prediction that in some circumstances animals might actually produce less free radicals when expending the most energy – most notably when the mitochondria are uncoupled and membrane potential is low. This theoretical postulate was confirmed observationally in mice when it was shown that across individuals it was those individuals with the highest energy metabolic rates that lived the longest, and such individuals had greater uncoupling of their muscle mitochondria [42]. In addition, DR does not appear to extend lifespan through reduced mass-specific metabolic rate, indeed it may actually increase total metabolic rate when account is made of detailed changes in body condition [43], although considerable debate still exists on how best to control for the reduced body mass and altered body composition that accompanies DR (for discussion see [44] and references therein).

The free-radical theory predicts that any intervention that increases protection against free radicals, decreases oxidative damage and will slow ageing and subsequently extend lifespan (and vice versa). Although the exposure of organisms to free-radical-generating chemicals and subsequent mortality has been cited as further support for the role of free radicals in ageing, more research is necessary to unequivocally answer whether this is indeed accelerated ageing per se [37]. The effect of antioxidants on lifespan in model organisms [45–47] and their effect on various health parameters in humans is unclear [48, 49], as is the efficacy of pharmacological mimetics of enzymes such as SOD (e.g. EUK-8; EUK-134; [37, 50]). Indeed, it has recently been suggested that the abrogation of free-radical signalling through antioxidant supplementation removes any positive health benefits derived from exercise in humans [51]. These same authors suggest that, contrary to the free-radical theory, an adaptive response in the mitochondria to increased free radical levels is necessary to induce a stress response, which ultimately enhances metabolic health and longevity; termed mitohormesis [52].

Genetic overexpression of various antioxidant enzymes, including SOD and catalase, have been shown to extend lifespan in *Drosophila* [53–55], and the effect of multiple antioxidant transgenes on lifespan appeared to be partially additive [55]. Human catalase overexpressed specifically in the mitochondria also extended lifespan in mice [56]. However, in contrast, comprehensive experimental studies performed by the Richardson and Van Remmen groups in San Antonio, Texas, systematically examining the augmentation or deletion of various antioxidant genes on lifespan, pathology and oxidative stress in mice did not support the free-radical theory [39, 57]. These researchers have shown that overexpression of various antioxidants did not increase lifespan in mice [57, 58]. In addition, they have also demonstrated that while MnSOD- and glutathione peroxidase-1-knockout mice had greater oxidative damage and a higher cancer incidence at 28-30 months of age, no negative effect on lifespan was seen [59]. In agreement, single or multiple deletions of various SOD isoforms in *Caenorhabditis elegans* did not shorten lifespan despite protecting against oxidative stress [37, 60, 61]. In fact, loss of SOD-2 both increased lifespan at the same time as it increased sensitivity to oxidative stress in this model organism [62]. The bottom line of these comprehensive studies is that knocking down the protection system, as anticipated, generally induces greater oxidative damage. However, what was unexpected was that such damage had absolutely no impact on the age-related mortality profiles of the animals in question. The mice seemed oblivious to the damage that occurred when lacking crucial elements of their antioxidant protection system.

These studies of genetically modified mice were undertaken within specific pathogen-free (SPF) conditions, and adherents of the oxidative damage life history theory took refuge in the assumption that perhaps there was something unusual about the laboratory mouse that made such observations irrelevant to ecology. However, more ecologically relevant information was not long in coming – and the news for the free-radical theory was not great. Selman et al. [63] maintained short-tailed field voles (*Microtus agrestis*) at 7 °C and examined their lifespan, antioxidant protection and oxidative stress levels compared to control animals kept at 22 °C. In contrast to the predictions of the free-radical theory, there was no effect of cold exposure on lifespan and only negligible effects on antioxidant protection and oxidative stress despite metabolic rate being significantly elevated in the cold. These results were replicated a year later in male mice that were not maintained under SPF conditions [64]. However, one could still always infer that these rodents were kept in unusual conditions, protected from predators and pathogens with ready access to a nutritious diet. Perhaps therefore the most surprising data refuting the free radical theory of ageing came from studies of the naked mole rat (NMR; *Heterocephalus glaber*) a eusocial rodent with a recorded lifespan in captivity of over 28 years, approximately eight times longer than similarly sized mice. It was thought that this remarkable longevity relative to other rodents may be explained by some extremely efficient mechanism to protect and/or repair against free radicals. Contrary to the predictions of the free-radical theory of ageing, NMRs combine a remarkably long-life with significantly elevated tissue levels of DNA, lipid and protein oxidative stress [65]. In addition, these animals do not have exceptionally high levels of antioxidant protection [66] and possess negligible hepatic cellular glutathione peroxidise activity and significantly reduced glutathione levels compared to mice. Finally, at the end of this long string of accumulating negative data, we have the small, blind, white olm with unexceptional energy demands and unremarkable mechanisms to cope with resultant oxidative stress – yet living exceptionally long lives.

Conclusion and perspectives

While there is a plethora of correlative studies particularly using model organisms in the laboratory that describe a link between free-radical damage to macromolecules and ageing, more direct tests, particularly those using targeted genetic manipulations are far less conclusive, and are now leading some to question the basic doctrine surrounding the free-radical theory. Ecological and laboratory-based studies using novel organisms are likely to be critical if we hope to understand the mechanisms underlying the aging process. Indeed,

several of these novel organisms, including the olm, have now struck a further blow to the free-radical theory. So is the free radical theory of ageing finally dead [39], or is it, as suggested by some authors, apparently invincible in the face of negative data [67]? It seems that if the gold standard by which the theory is evaluated is its role in determination of lifespan, then there is now a large body of evidence to question that role. However, it has been recently suggested that perhaps the lifespan effect is too harsh a criterion by which the theory should be evaluated. Rather the role of oxidative stress on organism health might be a more appropriate method to assess its importance [68]. This suggestion is perhaps simply a recognition that it was always unlikely that understanding a complex biological phenomenon like ageing would be achieved by such a simplistic hypothesis. Indeed, this more nuanced approach to the idea of free radicals and oxidative stress and their role in degenerative disease may be a much more sensible way to view the process of ageing, than a simple 'yes' or 'no' answer as to whether the free-radical damage hypothesis is correct or not. Indeed, even those most sceptical about the free-radical model have tended to agree that despite the negative evidence it seems likely that oxidative stress will play some sort of role in the ageing process. Certainly, there is an abundant amount of data indicating an association between elevated oxidative stress and increased risk of age-related diseases like various cancers, cardiovascular disease and neurodegeneration. Yet one factor marking out organisms such as amphibians like the olm and the NMR is their extremely low rates of cancer, in spite of their unexceptional defences against oxidative stress [69]. Consequently, the key to understanding the relationship of free radicals to degenerative disease and ultimately the process of ageing may not be obtained by trying to fathom whether animals have low or high rates of free-radical production, or even whether they sustain high or low rates of oxidative damage, but rather how they mitigate, or not, translation of this damage into age-related disease. Therein may lie the key questions for the future.

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