# Redox Metabolism and Longevity Relationships in Animals and Plants

Edited by
Christine H Foyer, Richard Faragher & Paul Thornalley

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# Abbreviations

| | |
|---|---|
| AASA | 2-aminoadipic semialdehyde |
| α-TQ | α-tocopherolquinone |
| ABA | abscisic acid |
| ABI4 | ABA-insensitive 4 |
| ACC | 1-aminocyclopropane-1-carboxylic acid |
| AD- and AR-EDMD | autosomal dominant and recessive forms of Emery-Dreyfuss muscular dystrophy |
| AF | atrial fibrillation |
| AGPase | ADP-glucose pyrophosphorylase |
| AKRd | aldoketo reductases |
| ALR | aldose/aldehyde reductase |
| ALT | alternative lengthening of telomeres |
| AOPPs | Advanced oxidation protein products |
| AP | activating protein |
| AP | Apetala 2 |
| APC | Adenomatous Polyposis Coli |
| APX | ascorbate peroxidase |
| ARE | antioxidant response elements |
| AS | arthropathy syndrome |
| ASH | ascorbate |
| ATM | Ataxia Telangiectasia Mutated protein |
| ATR | ATM and Rad 3-related |
| AWS | Atypical Werner's syndrome |
| BAP | Benzylaminopurine |
| BMP | bone morphogenic protein |
| bZIP | basic leucine zippers |
| CaBPs | calcium-binding proteins |
| CaM | calmodulin |
| CAT | Catalase |
| CBF | C-repeat binding protein |
| CCS | copper chaperone of SOD protein |
| CDK | cyclin-dependent kinase |
| CEL | $N_{\varepsilon}$-carboxyethyl-lysine |
| CGCs | cerebral giant cells |
| ChIP | chromatin immunoprecipitation |
| CMA | $N_{\omega}$-carboxymethylarginine |
| CMC | $N_{\varepsilon}$-carboxymethyl-cysteine |
| CML | $N_{\varepsilon}$-carboxymethyl-lysine |
| CMT2B1 | Charcot-Marie-Tooth syndrome type 2B1 |
| CoQ | ubiquinone |

| | |
|---|---|
| CPs | cysteine proteases |
| c-PTIO | 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide |
| CR | caloric restriction |
| CRM | exportin chromosome region maintenance |
| C/R ratio | calorimetric/respirometric ratio |
| CRY | cryptochrome |
| DBMIB | 2,5-dibromo-3-methyl-6-isopropyl-p-benzoquinone |
| DCM-CD | dilated cardiomyopathy with conduction system disease |
| -CF | DCM with early-onset cardiac fibrosis |
| -VA | DCM with apical left ventricular aneurysm |
| DCMU | 3-(3,4-dichlorophenyl)-1,1-dimethyl urea |
| 3DG | 3-deoxyglucosone |
| 3DG-H | $N_{\delta}$-(5-hydro-5-(2,3,4-trihydroxybutyl)-4-imidazolon-2-yl)-ornithine |
| DHS | dropped head syndrome |
| DMEM | Dulbecco's modified Eagle's medium |
| DMQ9 | demethoxy-ubiquinone-9 |
| DNA-PK*cs* | DNA-dependent protein kinase catalytic subunit |
| DOD | 2-deoxy-D-glucose |
| DR | dietary restriction |
| DSBs | DNA double-strand breaks |
| E | Enzyme |
| EEE | excess excitation energy |
| EDMD | Emery-Dreyfuss muscular dystrophy |
| EDS1 | enhanced disease susceptibility 1 |
| EIN2 | ethylene insensitive 2 |
| EL | excess light |
| ER | endoplasmic reticular/reticulum |
| ESI | electrospray ionization |
| ESTs | expression sequence tags |
| ETC | electron transport chain |
| FAD | flavin adenine dinucleotide |
| FCCP | carbonyl cyanide p-trifluoromethoxyphenylhydrazone |
| FL | $N_{\varepsilon}$-fructosyl-lysine |
| FPLD | Dunnigan's familial partial lipodystrophy |
| FTI | farnesyl transferase inhibitor |
| γ-ECS | γ-glutamyl cysteine synthetase |
| GA | gibberellins |
| GAPDH | glyceraldehyde 3-phosphate dehydrogenase |
| GBF1 | G-box binding factor 1 |
| GC-MS | gas chromatography with mass spectrometric detection |
| GFP | green fluorescent protein |
| G-H1 | $N_{\delta}$-(5-hydro-4-imidazolon-2-yl)-ornithine |
| GID1 | gibberellin-insensitive dwarf 1 |
| Glo1 | glyoxalase 1 |
| GPX | glutathione peroxidase |

| | |
|---|---|
| GSA | glutamic semialdehyde |
| GSH | reduced glutathione |
| GSSG | glutathione disulphide (oxidized glutathione) |
| GSTs | glutathione S-transferases |
| HACA | 6-hydroxy-2-aminocaproic acid |
| HAVA | 5-hydroxy-2-aminovaleric acid |
| HMG-CoA reductase | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase |
| HGPS | Hutchinson-Gilford progeria syndrome |
| 3-NHA | 3-nitro-4-hydroxyphenylacetic acid |
| HNE | 4-hydroxynon-2-enal |
| $H_2O_2$ | hydrogen peroxide |
| HPLC | High performance liquid chromatography |
| HR | hypersensitive disease response |
| HSF-1 | heat shock factor 1 |
| HSP | Heat shock protein |
| hTERT-BMCs | telomerase-immortalized bone marrow cells |
| HXK1 | Hexokinase 1 |
| IAA | Auxin |
| IAF | 5′-iodoacetamide fluorescein |
| IF | intermediate filament (proteins) |
| IGF | Insulin-like growth factor |
| IIS | insulin/IGF-1 signalling |
| INM | inner nuclear membrane |
| JA | jasmonic acid |
| JNK | Jun N-terminal kinase |
| KFA | kynurenine formamidase |
| KO | knockout |
| Kyn | kynurenine |
| LAP2α | lamina-associated polypeptide 2α |
| LC-MS | liquid chromatography with mass spectrometric detection |
| LFA | Lethal foetal akinesis |
| LGMD1B | limb girdle muscular dystrophy with atrioventricular conduction disturbances type 1B |
| LL | Low light |
| LMNA | lamin A/C |
| LMNA | lamin A gene |
| LMNA | lamin B gene |
| LSD1 | lesion simulating disease 1 |
| MAD | mandibulo-acral dysplasia |
| MALDI | matrix-assisted laser desorption/ionization |
| MAPKs | mitogen-activated protein kinases |
| MAPKKKs | MAPK kinase kinases |
| Met | methionine |
| MetSO | methionine sulphoxide |
| MG | methylglyoxal |
| MG-H1 | $N_\delta$-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine |

| | |
|---|---|
| Msr | methionine sulphoxide reductase |
| mtDNA | mitochondrial DNA |
| NAA | naphthaleneacetic acid |
| NAC | *N*-acetyl-cysteine |
| $NADP^+$ | Nicotinamide adenine dinucleotide phosphate |
| NADPH | Nicotinamide adenine dinucleotide phosphate (reduced form) |
| NCEs | new chemical entities |
| NE | nuclear envelope |
| NF | nuclear factor |
| NFK | N-formyl-kynurenine |
| NHEJ | non-homologous end-joining pathway |
| NahG | bacterial salicylate hydroxylase |
| NLS | nuclear localization sequence |
| NNT | nicotinamide nucleotide transhydrogenase |
| NO | nitric oxide |
| NPCs | nuclear pore complexes |
| NPQ | non-photochemical quenching |
| NPR1 | Non-Expressor of Pathogenesis Related Genes 1 |
| NR | nitrate reductase |
| NT | nuclear transfer |
| 3NT | 3-nitrotyrosine |
| ONM | Outer nuclear membrane |
| OXI1 kinase | Oxidative signal-inducible 1 kinase |
| PAD4 | phytoalexin deficient 4 |
| PAGE | Polyacrylamide gel electrophoresis |
| PBL | peripheral blood leucocyte |
| PCD | programmed cell death |
| 3PGA | 3-phosphoglycerate |
| PGlyco | phosphoglycolate |
| PHOT | phototropin |
| PHY | phytochrome |
| PIF3 | phytochrome-interacting transcription factor 3 |
| PM | peripheral membrane |
| PMSR | protein methionine sulphoxide reductase |
| POS | polycystic ovaries with type-A insulin resistance syndrome |
| PP2C | protein phosphatase 2C |
| PR | Pathogenesis-related |
| PSI/PSII | photosystems I and II |
| PUFAs | polyunsaturated fatty acids |
| QM-DCM | quadricipital myopathy with dilated cardiomyopathy |
| QTLs | Quantitative trait loci |
| Rbcs | RuBisCO small subunit |
| Rboh | Respiratory burst oxidase homologue |
| RD | restrictive dermopathy |
| Rel | reticuloendotheliosis viral oncogene |
| RGR | relative growth rate |
| RNAi | RNA interference |

| | |
|---|---|
| RNS | reactive nitrogen species |
| ROS | reactive oxygen species |
| R-PCD | runaway programmed cell death |
| RuBisCO | ribulose bisphosphate carboxylase/oxygenase |
| RuBP | ribulose-1,5-bisphosphate |
| SA | Salicylic acid |
| SAA | Systemic acquired acclimation |
| SAG | Senescence-associated gene |
| SAGs | senescence-associated genes |
| SAPK | stress-activated protein kinase |
| SAR | Systemic acquired resistance |
| SCF | Skp, Cullin, F-box-containing complex |
| SDH | succinate dehydrogenase |
| SIPS | stress-induced premature senescence |
| SNAP | S-nitroso-*N*-acetylpenicillamine |
| SNF1 | sucrose non-fermenting-1 |
| SN | sodium nitroprusside |
| SNP | single-nucleotide polymorphism |
| SnRKs | SNF1-related protein kinases |
| SOD | superoxide dismutase |
| SR | sarcoplasmic reticulum |
| SREBP1 | sterol-regulatory element-binding protein |
| STASIS | stress- and stimulation-induced senescence |
| TCA | Tricarboxylic acid cycle |
| TCF/LEF | T cell factor/lymphoid enhancer factor |
| TDO | tryptophan 2,3-dioxygenase |
| TGF | tumour growth factor |
| TOF | train of four |
| trp | tryptophan |
| TRX | thioredoxin |
| UCPs | uncoupling proteins |
| UPLC | ultra-high-performance liquid chromatography |
| VSP | vegetative storage protein |
| Wnt | Wingless/INT |
| WRN | Werner's protein |
| WS | Werner's syndrome |
| WT | wild type |

# Preface

The massive social and economic changes that occurred during the last century not only led to the virtual eradication of nutrient deficiency diseases in the developed world, but they also focused attention on the problems associated with the appearance of a relatively new phenomenon, an increasingly ageing population. In the last two decades, scientists studying the animal and plant kingdoms have become increasingly occupied with gaining a deeper understanding of the genetic basis for ageing and the impact of the environment on this process. Since, the maintenance of youth, youthfulness or at least healthy ageing in animals is a goal aspired to by many, researchers in this area have become increasingly engaged in developing innovative strategies for delaying the ageing process. Similarly, plant stay-green phenotypes are highly sought after because of their enhanced performance in the field, particularly during periods of exposure to environmental stress. This volume addresses the recent issues that have emerged in ageing research, outlined in the context of the individual personal perspectives and insights of key researchers working in the field.

Current knowledge would suggest that in all probability, the mythical Fountain of Youth resides in the ability of animals and plants to recycle damaged cells. While the complex and multifactorial aetiology of the ageing process remains far from understood, a consensus of view is emerging regarding the nature of the ageing process as it operates in the few model animal species that have been subjected to intensive study. The original simple concept defined in the 'oxidant theory of ageing', of a clear causal relationship between oxidative damage and animal ageing, has not been substantiated and is giving way to a much more complex and intellectually stimulating picture of ageing as a failure to recycle damaged cells and macromolecules. All aerobic organisms exploit the potential of oxygen chemistry and effectively deal with the ever-present generation of reactive oxygen species (ROS) and reactive nitrogen species (RNS). A late addition to this grouping of reactive metabolites is sugar-derived dicarbonyls involved in physiological glycation. Within the context of the sugar economy of the cell, redox metabolism impacts directly on genome and proteome, which are modified by processes such as oxidation, nitration and glycation. The field of redox biology has witnessed a reappraisal of the function of ROS and antioxidants in recent years. Once considered only as 'molecular hoodlums' to be suppressed or policed by the antioxidant system, ROS are now considered to be dynamic information-rich signalling molecules in some instances and mediators of oxidative damage in others. Similarly, taking low-molecular-weight antioxidants such as vitamins C and E as dietary supplements is no longer considered to be beneficial in retarding the ageing process. This volume reviews current concepts concerning cellular redox homeostasis and ageing in animals and plants, relationships to programmed cell death, the production of oxidants and dicarbonyls, the ways that different organisms perceive and respond to oxidative, nitration and glycation challenges, and how this might be intricately connected to ageing and lifespan. The topics to be covered in this volume include the science of ageing, the

ecology of ageing, the role of mitochondria and energy metabolism, the free radical theory of ageing, redox homeostasis and antioxidant signalling, reactive oxygen and reactive nitrogen species, senescence and programmed cell death, glycation, quantifying protein and nucleotide modifications during ageing, redox proteomics in ageing in animals and plants, genomics of ageing and caloric restriction, bioinformatics tools in human ageing genomics and caloric restriction mimetics. The chapter by Richard Faragher provides an overview of ageing in animals, while the chapter presented by Paul Thornalley and co-investigators provides a critical appraisal of methods used to assess damage to the proteome in ageing, and considers the emerging evidence for a decline in enzymatic defences against oxidation, nitration and glycation with age as an explanation for increased proteome damage of the ageing phenotype. The protective effects of glyoxalase 1 in plants exposed to stress are considered in the chapter by Sudir Sopory. An anti-ageing effect of over-expression of glyoxalase 1 in *Caenorhabditis elegans* (see cover) was recently reported by Thornalley and collaborators. While concepts of lifespan are rarely considered in plants, the possible roles of oxidants in leaf senescence are discussed by Ulrike Zentgraf and in programmed cell death in seeds by Paul Bethke. The roles of ROS as signalling molecules in plants are discussed in detail by John Hancock, who considers the paradox between their functions in the stimulation of growth and development and as triggers for programmed cell death. The role of cellular redox metabolism in the developmental responses of plants to enrichment in atmospheric carbon dioxide are described by Christine Foyer and colleagues, while the chapter from Stanisław Karpiński and his team considers the interfacing pathways of light signalling and plant/pathogen responses in programmed cell death.

The chapter by João Pedro de Magalhães considers informatics and data approaches, providing a database of genes related to human ageing (GenAge) and related ageing research informatics (http://genomics.senescence.info/index.html). The battery of protective genes that are involved with detoxification of xenobiotics and the enzymatic defences against protein damage, and that are associated with longevity in *C. elegans*, is reviewed in the chapter by David Gems. The latest research on increased longevity through dietary restriction and therapeutic agents with related mimetic activity is discussed by Stephen Spindler, who demonstrates that the calorie-restricted state in mammals is associated with significant tissue re-sorption and the up-regulation of enzymes involved in phase 1 and phase 2 drug metabolism – the same pathways associated with extended lifespan in the simple metazoan organisms. Finally, Lamb and Shiels provide an updated review on the role of telomere biology in ageing at the cellular level while Pekovic and Hutchison put forward a cellular stress hypothesis to explain the links between A-type lamins, ageing and premature ageing diseases.

These chapters bring together current knowledge on cell senescence and programmed cell death in animals and plants with the underpinning processes of tissue turnover and molecular recycling. While Ponce de Leon's never-ending search for the mythical Fountain of Youth proved to be fruitless, the contents of this volume demonstrate that we are now in a better position to understand the respective roles of accumulating damage to protein and nucleotides, and thus develop nutritional and prospective interventions to slow the ageing process.

June 2008. Christine Foyer, Richard Faragher & Paul Thornalley

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# What can we learn from the cross-species biology of ageing?

Richard G.A. Faragher

## 1 Introduction: What is ageing and why should we care?

In 1967, the Society for Experimental Biology played host to a symposium on the biology of ageing which included studies of the comparative biology of ageing in plants, insects, protozoa and mammals (Woolhouse *et al.*, 1967). Scientists lacking an all-consuming interest in the field were less than impressed. One participant in particular was heard to remark that 'a professional biochemical team could have all this sorted out in a fortnight' (Woolhouse *et al*., 1967). Four decades on, this chapter is written with much the same intent as that original SEB volume. It is a newscast-like account of the longest fortnight in scientific history (or perhaps 10 days since many areas in ageing research remain opaque) written for the student who is embarking on a career as a gerontologist or the specialist who has, perhaps by chance, blundered into the minefield of confused terminology and muddled debate that until recently characterized ageing research.

The thing which is most striking as a contemporary gerontologist looking back at the literature from the beginnings of the field is the complete absence of any organizing theoretical principles which would allow investigators to gain understanding (rather than simply experimental results) from the systems they chose to study. There seems to have been little consensus on what the ageing process actually was and this rendered discussion of any subsequent questions turbid to say the least. Some years before, Strehler (Strehler and Mildvan, 1960) had set out four principle criteria that defined the ageing process (or at least distinguished it from maturation and development) but there is little evidence from the 1967 SEB symposium that these were enthusiastically embraced by his contemporaries. Strehler defined ageing as a process which was:

- Universal (i.e. all members of a population of organisms will show it, a distinction from infectious disease);
- Progressive (the process was continual and incremental rather than sudden as in the case of 'programmed' organismal death);
- Intrinsic (distinguishing ageing from death due to outside events);
- Degenerative (this captures the idea that ageing is associated with both increasing chances of mortality but also an increasing level of morbidity).

Applying these criteria helps distinguish ageing from other pathological processes at work within organisms. However, they do not lay sufficient stress on one key aspect of the physiology of ageing, the increased frailty of old organisms compared to their young counterparts. Put simply, aged organisms often fail to survive physiological stresses which young organisms are able to weather effectively. For example, the budding yeast *Saccharomyces cerevisiae* reproduces asexually but shows an ageing process marked by the eventual cessation of reproductive capacity in older mother cells (Powell *et al.*, 2003). If young and old cultures of yeast are exposed to an ageing that induces physiological stress (such as UV radiation), old yeast cells are markedly more prone to die (Kale and Jazwinski, 1996). In the nematode *Caenorhabditis elegans*, multiple mutations or interventions that lengthen the life of the animal also impart a stress resistance phenotype compared to wild-type controls (Gill *et al*., 2003). Many other examples of similar phenomena across the biosphere could be listed.

Understanding and dealing with human frailty, along with human morbidity, are both the major challenges facing modern gerontology and its primary justification as more than a disinterested search for the truth concerning our condition. Morbidity, the time spent sick before either death or recovery occurs, is prolonged, painful, expensive and undesirable for all concerned (it has been calculated that a 1% reduction in morbidity will result in savings of billions of pounds per year in long-term care costs by the middle third of this century). In contrast, death as a result of frailty can be both abrupt and apparently cheap (as in the case of death by infection in elderly humans following hip fracture); however, the fact that it falls on members of the population who are healthy, active and socially engaged means that it also has significant financial, as well as emotional, costs.

To venture upon a mechanistic analogy, ageing is the study of the biology of worn parts and the consequences thereof. But, before discussing the mechanisms of wear in more detail, it is necessary to consider briefly a question which has troubled many thinkers in human history: 'why does ageing happen at all?'

## 2 Why does ageing happen?

Ageing is not universal across all species, or even among all metazoans, but it is extremely common and thus presumably provides some form of selective advantage to organisms that show it compared to those that do not. The theories I am about to present concerning ageing have been formulated with regard primarily to species that show a germ-line to soma distinction (the soma is the bits of you that are reading this chapter). Although a soma could, in principle, be either ageing or non-ageing the germ-line must, by definition, be immortal in order to allow the organism to contribute any offspring at all to future generations (an observation that appears to have been first made by the 19th-century evolutionary biologist August Weismann). These theories thus have only a tangential bearing on organisms that can reproduce clonally for extended periods (e.g. some plants). Nonetheless, at least some asexual metazoans do display an individual ageing process (Martínez and Levinton, 1992) and clonal lineages of these organisms can suffer a reduction of average fitness through time by the accumulation of slightly deleterious mutation which cannot be repaired without sexual recombination (a mechanism known as Muller's Ratchet). Muller's Ratchet should, in theory, drive purely asexual replicators to extinction unless the population is extremely large.

However, many metazoan species that replicate clonally retain the ability to initiate sexual reproduction at specific time points (often generating germ-line from undifferentiated stem cells). Such organisms can thus be seen as opting in and out of the germline/soma distinction and when they are 'in' the evolutionary forces described below most definitely bite. The best current example of this is probably the cnidarian *Hydra*, which has been shown to be non-ageing in its asexual form (Martinez, 1998) but after sexual reproduction the population shows an exponential increase in mortality rate associated with functional deficits in cellularity, contractile movement and food capture in individual organisms (Yoshida *et al.*, 2006).

Modern explanations for 'why ageing happens' are all based on the evolutionary truism that the force of natural selection declines with age. This means that, even in a population of immortal organisms, there are always far fewer chronologically old ones than young ones around (because the longer a given organism has been around the more likely it is to have been eaten, met with an accident, etc.). Thus, even though the reproductive ability of 'old' and 'new' non-ageing organisms is the same, the 'old' organisms contribute fewer offspring to the next generation than the 'new' organisms *simply because there are fewer of them*. Thus, any mutation that favours early life fecundity will be selected for even if it results in deleterious effects later on in the lifetime (a type of gene action termed antagonistic pleiotropy; Williams, 1957). This view of ageing argues against the operation of a 'programme' controlling the ageing of individuals (i.e. the existence of a genetic pathway or process that causes the organism to age but does nothing else). Rather, it suggests that ageing will result from an accumulation of unrepaired faults, which are generated at different rates in different tissues.

Antagonistic pleiotropy and related theories which conceptualize ageing as the by-product of selection for early-life fecundity (such as the 'disposable soma' theory of Thomas Kirkwood, which considers the problem in terms of resource allocation between somatic repair and reproduction) have proved highly satisfactory in explaining why ageing happens (Kirkwood, 2005). According to antagonistic pleiotropy, optimal lifespan is determined by selection pressure for maximum reproductive success. This is itself determined by the level of environmental risk (e.g. the chance of predation) to which members of the population are exposed as a consequence of the ecological niche which they occupy. The huge range of lifespans seen among organisms on this planet can thus be conceived of as points lying on a continuum between two opposing evolutionary strategies. Organisms at very high levels of risk tend to display 'prodigal' (or *r* selected) life history strategies marked by single reproductive events which generate many small and rapidly maturing young which then disperse without parental care. In contrast, species following 'prudent' (or *K*-selected) strategies tend to reproduce steadily and produce a few large young that receive intensive parental care and mature slowly. Clearly, prudence is only an option for a species whose members have a chance of making it to the end of the week in one piece. These models allow an organism to be non-ageing only under two conditions: (i) the lack of a germ-line soma distinction (exemplified by non-sexually differentiated *Hydra* discussed above) or (ii) if the efficiency of the organism at producing offspring per unit energy increases over time (thus counteracting the beneficial effect of mutations favouring early fecundity).

Fecundity-based theories have also provided a powerful conceptual framework for the manipulation of lifespan itself. This is best exemplified by the work of Charlesworth, Rose and colleagues (Rose and Charlesworth, 1981). These researchers

were able to extend significantly the overall longevity of female *Drosophila* by the simple expedient of selecting those females that showed the highest fecundity in the late phase of the lifespan (typically 20–25 days) as the parents of the $F_1$. If performed over many generations this procedure produces increased longevity, increased late-life fecundity and generally enhanced resistance to physiological stress compared to age-equivalent wild-type flies (Nghiem *et al.*, 2000). Essentially similar findings have been reported by Austad, comparing populations of opossums living in relatively high and low predation environments. These studies are consistent with the hypothesis that selection for early-life fecundity is the primary reason for organismal ageing.

Despite these successes, care should be taken always to view antagonistic pleiotropy and disposable soma as theories or hypotheses (q.v.) not immutable laws delivered from on high. The number of species in which their predictions have been tested is extremely limited and the majority of these have been organisms maintained under laboratory conditions for many generations. It has even been suggested that these hypotheses may have actually hindered progress in evolutionary studies of ageing research (Promislow and Pletcher, 2002). This question will be revisited at the end of this chapter.

## 3 How does ageing happen?

Johnson once famously described patriotism as the 'last refuge of the scoundrel' and in a similar vein gerontology could sometimes be described as the last refuge of the theorist. At the last count, there were approximately 300 distinct 'theories of ageing' (Medvedev, 1990). Some gerontologists (often new students) see this as evidence of a flourishing and vigorous field. However, like many of my colleagues, I do not see this morass in a positive light; after all, no-one in the mitochondrial field now hankers for the good old days when the chemiosmotic hypothesis stood alongside rival theories (including the production of the elusive high energy '~' and conformational strain) as an attempt to explain how ATP was produced. The recognition that proton-motive force generates ATP allowed the field to move forward. Thus a field with a few well-tested ideas is a field which is going places. A field which lovingly retains many theories (some of them discredited antique curios fit only for the historian of science) probably deserves the jibe made about archaeology (sometimes called by wags the 'science of rubbish'): 'As fast as the rubbish is dug up; it is written down'. I would advise anyone new to the field of ageing to treat many theories of 'how we age' as though they carried a health warning.

To be fit for purpose as a research tool any scientific hypothesis must satisfy the following criteria (best described by Karl Popper). To avoid the fundamental philosophical problem posed by induction (the commonplace but unprovable assumption that objects outside the study will behave in the same way as objects within the study) any theory must be capable of being refuted or falsified (i.e. it should make predictions that are incompatible with certain possible results of observation). Thus all good scientific theories should be very precise (to maximize the number of potentially incompatible observations that could be made) and every genuine test of a hypothesis should be an attempt to falsify it (because it is easy to obtain verifications of a theory if you do nothing but look for them). In addition, every theory formulated to explain ageing needs to meet three common-sense criteria.

- In order to cause organismal ageing the postulated mechanism must be present *in vivo*.
- If the mechanism is present *in vivo* it must be capable of exerting degenerative effects.
- Increasing the rate at which the mechanism operates should increase the rate of ageing and (perhaps more convincingly) slowing the rate at which the mechanism works should slow the rate of ageing.

Looked at from this point of view very many theories of ageing (e.g. the original formulation of the somatic mutation hypothesis) either collapse due to insufficient precision (they are simply untestable) or have already been tested and refuted. The classic example of the latter is probably Orgel's error-catastrophe theory (Orgel, 1963). This theory proposed that insertion of the wrong tRNA into a growing polypeptide chain during translation could give rise to long-lived molecules (such as DNA or RNA polymerase) which were themselves error-prone. This was postulated to drive an exponential propagation of 'errors' through the cell leading to cell death. The most elegant refutation of this is probably a study in which senescent cells (q.v.) were infected with a virus that used the host replication machinery in order to reproduce. If 'error-catastrophe' had occurred, this machinery should have been dysfunctional[1]. In fact, it gave rise to yields of virus essentially identical to those found in non-senescent cells from the same donors (Danner *et al.*, 1978). Unfortunately, well-designed experiments of this type have been uncommon in ageing research until quite recently.

Attempts to arrive at a mature understanding of the cross-species biology of ageing also run the risk of foundering on an unusual problem in biology. Cross-species studies have been hugely successful in elucidating the operation of a very wide variety of fundamental processes (e.g. DNA replication and repair) because the molecular machinery necessary to carry out the process under study is often tightly conserved (i.e. it is a safe bet that a DNA polymerase from *E. coli* will be substantially similar to that found in *Homo sapiens* because both species ultimately derive from a common ancestor and the functional requirements of replicating human and bacterial DNA are very similar indeed). There has thus been an expectation in some quarters of close similarities between species when the mechanistic causes of ageing are the subject of investigation (i.e. the study of ageing in yeast will inform on human ageing). To an extent this expectation has been justified. A series of mutants in genes affecting the insulin-IGF axis have been shown to lengthen lifespan in very different species by mechanisms that have yet to be fully clarified (Partridge *et al.*, 2005). Unfortunately, there is no logical reason that this must always be the case.

Ageing is the unprogrammed result of selection for early reproductive success and thus there is no *a priori* reason why the deleterious changes that result from that selection pressure have to be universal across species (by the same token there is also nothing to exclude similar changes if they impact a common pathway). In fact, there

[1] In retrospect, it probably wasn't worthwhile carrying out this experiment at all since error catastrophe predicted cell death and senescent cells were known by this time not to do this at appreciably greater frequencies than their growing counterparts. However, in fairness the theory *was* concerned with cells in culture and *was* being invoked to explain senescence at the time (e.g. Holliday and Tarrant, *Nature* **238:** 26–30).

is evidence that the primary driver of organismal ageing can sometimes be quite different between species. For example, a major cause of ageing and death in female *D. melanogaster* is the toxic effect of compounds present in the seminal fluid products secreted from the main cells of the male fruit fly accessory gland (Barnes *et al.*, 2008). Seminal fluid toxicity is not seriously advanced as a primary cause of mammalian ageing. Similarly, replicative senescence (the loss of divisional capacity in the mitotic tissue compartments of the soma) is not seriously advanced as an ageing mechanism for species such as *C. elegans*, that have a completely post-mitotic soma. In summary, an ageing mechanism which is 'universal' is not necessarily 'primary' in all species and a mechanism which is 'primary' in one species is not necessarily 'universal' in all the others. Picking one's way through this morass requires clear thought and by way of illustration I give two theories which stand out from the confusion. One is a much-touted theory which (in its original form) probably captures everything that used to be wrong with studies of 'how ageing happens' but which is now being reformulated in a much more testable manner. The other is a much-maligned theory that does seem a candidate for a plausible ageing mechanism in some species at some times.

### 3.1 *Who's afraid of 'free' radicals?*

The free radical theory of ageing proposes that damage caused by free radical reactions is a key contributor to both ageing and age-associated disease. It was first proposed by Denham Harman in the 1950s (Harman, 1956) and is probably the only theory of ageing that the general public are familiar with. Perhaps no single theory in gerontology has attracted quite as much interest among researchers.

The free radical theory passes two of the three common-sense criteria I gave above. Free radical reactions certainly take place in real bodies (a fact not formally established at the time the theory was proposed) and both radicals and reactive oxygen species clearly have the potential to exert degenerative effects. However, the evidence that slowing the rate at which oxidative damage occurs slows the rate of ageing is far more problematic. Much of the data on which the oxidative damage theory of ageing is based is correlative and is based on the examination of antioxidant defence levels in different species to determine if the ability to defend against oxidative stress correlates with species-specific lifespan (in general the correlation is rather good but see Section 4.0). Similarly, long- and short-lived strains of the same organism have been compared for antioxidant defence levels (longer-lived animals have more; see Dudas and Arking, 1995). However, when interventional tests of the theory have been attempted (typically by feeding ageing rodents dietary antioxidants such as vitamin E to try and extend their lifespans) the results have been far less successful. One particularly well-conceived series of studies may stand for the whole. A class of molecules known as the salens were shown some years ago to act as superoxide dismutase and catalase mimics *in vivo* and *in vitro*. In particular these molecules were shown to be able to somewhat extend the lifespan of mice carrying a loss-of-function mutation in mitochondrial superoxide dismutase (these animals normally have a lifespan of only a few days, the salen was able to increase this to some weeks; Melov *et al.*, 2001). An initial report also appeared to show a clear increase in normal nematode lifespan when treated with the same compounds (Melov *et al.*, 2000). However, a subsequent series of nematode studies: (i) determined the dose of salen needed to give a protective effect against

paraquat-mediated killing (to ensure physiological efficacy) and (ii) measured the effect of continuous supplementation at that dose on the mean of maximum lifespans of a population of nematodes. No extension of lifespan could be observed (Keaney and Gems, 2003; Keaney *et al.*, 2004). This is quite a typical pattern in which a study seeking to test the free radical theory reports a positive effect and others (often in less commonly read journals) fail to reproduce it. Explanations for this failure to produce a consistent lifespan extension effect by interdiction of a pathway that is proposed to be of primary (and perhaps universal) importance generally include some form of the notion that the effective dose of antioxidants delivered is not high enough. Whilst there can be a certain amount of truth in these statements it is hard not to consider them a variant of the 'conventionalist twist'[2].

In at least one species (*C. elegans*) the classical formulation of the free radical theory of ageing appears to have been refuted. Knocking out the manganese-containing forms of the antioxidant enzyme superoxide dismutase has no apparent effects on lifespan (although loss of major Cu/Zn SOD, *sod-1*, does shorten life). Over-expression of *sod-1* does not produce any increase in longevity in this organism. These data are simply not consistent with a simple relationship between free radical (or oxidative) damage and organismal ageing. What is going on?

One clue is provided by microarray analysis of genes regulated by insulin/insulin-like growth factor-1 (IGF-1) signalling in this species. Mutants in this pathway significantly increase lifespan and that increased lifespan is associated with up-regulated antioxidant defences (including superoxide dismutase levels). However, many other enzymes are also up-regulated which have no role in dealing with oxidative stress but instead are components of either the phase 1 or phase 2 detoxification system used for xenobiotic metabolism. Thus it appears that long life is associated with increased activity in pathways that are primarily designed to detoxify a broad spectrum of damaged macromolecules. Molecules damaged by reactive oxygen species (ROS) are only one feedstock going into this recycling plant. This 'green theory' of ageing (Gems and McElwee, 2005) represents a considerable conceptual advance on anything which has gone before and is consistent with a number of other studies (not least the observation that over-expression of glyoxalase I increases lifespan in *C. elegans*; see Morcos *et al.*, 2008).

### 3.2 *Replicative senescence causes ageing: Definitely maybe*

Replicative senescence is a permanent block to further replication in cells from the mitotic tissue compartments of a metazoan organism. First reported in the 1960s by Leonard Hayflick and Paul Moorhead (reviewed in Faragher and Kipling, 1998) the phenomenon has been extensively studied in cells from mammals but much less so in cells from lower vertebrates (although it is known to occur in amphibians and may occur in molluscs). Senescent cells are normally unable to traverse the cell cycle as the result of an active block to cell-cycle traverse. This block usually occurs in the $G_1$

[2] According to Popper, conventionalist twists are the introduction of *ad hoc* assumptions into a theory by proponents when the original form of the theory appears to have been falsified by experiment. This rescues the theory from refutation at the price of lowering (or perhaps destroying) its scientific merit. Not all twists do this. It is perfectly acceptable to modify a theory provided the 'twists' themselves give rise to falsifiable predictions.

phase of the cell cycle and is mediated by one or more cyclin-dependent kinase inhibitors. In most circumstances the result is a living, viable and metabolically active, but reproductively sterile cell. The primary physiological role of entry into senescence is almost certainly to act as a longevity assurance mechanism by preventing the unconstrained proliferation of clones of cells that may have accumulated pro-carcinogenic mutations. Senescent cells are responsible for the decline in growth potential of *in vitro* cell populations that have undergone substantive turnover. They display many biochemical features that are distinct from their growing counterparts as a result of highly selective changes in gene expression. The expression of some genes goes up (as a consequence of both increased transcription and mRNA stabilization), the expression of others goes down and the expression of still more is unaffected. The overall effect is to produce a cell that is locked into a pro-inflammatory phenotype by comparison to cells of the same time that are still capable of division. It has been proposed that progressive tissue turnover during life leads to ever-increasing numbers of cells entering the senescent state.

For many years replicative senescence was a remarkably poor candidate as an *in vivo* ageing mechanism (measured against the three common-sense criteria above). From the 1960s to the 1990s, there was very little evidence that senescent cells were present in bodies. There was even less evidence that senescent cells were capable of exerting degenerative effects. The best evidence that there might be correlations between senescence and organismal ageing were probably: (i) the observation that the proliferative capacity of fibroblasts co-varied in a roughly linear fashion with maximum lifespan in eight different mammalian species (Röhme, 1981) and (ii) reports that fibroblasts derived from humans with progeroid (accelerated ageing) syndromes, such as Werner's syndrome and progeria, showed a greatly attenuated capacity to proliferate in culture (Kipling *et al.*, 2004). This was hardly impressive, but the lack of data represented a lack of interest in these questions. The majority of workers in the field were far more concerned with dissecting the molecular mechanisms that led to the initiation and establishment of the senescent state *in vitro*.

However, the situation is now very different. A landmark paper in the mid-1990s used a modified catalytic histochemical assay for β-galactosidase to demonstrate that senescent cells were present *in vivo* and that they increased in an age-dependent manner (Dimri *et al.*, 1995). Techniques for the visualization of senescent cells have improved considerably since this paper was published (although it is still far from easy) and a recent study using multiple markers has concluded that upwards of 15% of cells in the skin of old baboons are in fact senescent (Herbig *et al.*, 2006). Thus, there is limited but solid evidence consistent with senescent cells being present in real bodies.

When incorporated into reconstituted human skin equivalents, human dermal populations made senescent *in vitro* are known to increase dermal fragility and subepidermal blistering in a cell-number-dependent manner demonstrating that the presence of such cells could exert degenerative effects in *ex-vivo* equivalents (Funk *et al.*, 2000). However, a still more striking demonstration that senescent cells can produce life-threatening pathology was provided by experimental induction of senescence in living rat carotid arteries (Minamino *et al.*, 2004). This produced severe vascular inflammation and changes consistent with the development of atheroma. This study is probably the best current evidence that senescent cells are deleterious to living organisms.

Since the early 1990s, it has been known that the short replicative lifespan of Werner's syndrome is due to a three- to fivefold increase in the rate at which cells exit the cell cycle and become senescent *in vitro* (Faragher *et al.*, 1993). Although this strongly suggests that a similar process drives the accumulation of senescent cells in Werner's patients the best evidence that senescent cells cause accelerated ageing comes from work on two strains of mice made null for the Werner's syndrome gene (*wrn*). Mice singly deficient for *wrn* show little by way of any obvious premature ageing phenotype despite the fact that they show other cellular phenotypes of human WS. However, the differences in replicative lifespan between wild-type and $wrn^{-/-}$ mouse fibroblasts were small. However, a second knock out strain (a $wrn^{-/-}$ $terc^{-/-}$ double null) developed age-dependent pathologies that closely paralleled those seen in Werner's syndrome humans. These include grey hair, osteoporosis, type-II diabetes, cataracts, an elevated frequency of non-epithelial malignancies and premature death. Fibroblasts from the $wrn^{-/-}$ $terc^{-/-}$ animals showed accelerated replicative senescence *in vitro* (Kipling *et al.*, 2004). The conclusion that senescent cells are driving the 'ageing' changes is very hard to avoid.

## 4 Strengths and weaknesses of a cross-species approach

### 4.1 *Short-lived ageing models*

In a sense, the majority of studies of the ageing process are cross-species studies by inference. After all, there is limited utility in learning about the mechanisms of ageing in *C. elegans* or *Drosophila melanogaster* unless the data gained are eventually of value in advancing our understanding of human ageing. However, we may perhaps distinguish between three styles of cross-species study.

- The study of short-lived organisms that show a defined ageing process.
- The study of ultra-long-lived species that still show ageing.
- Comparative studies across a wide range of different species intended to test a mechanistic or evolutionary hypothesis.

The first of these styles of study is quite familiar. The use of organisms such as *C. elegans* and *Drosophila* has brought much needed rigour to biogerontology. The primary advantages of these species are that they reproduce quickly, have lifespans of only a couple of months and have excellent genetic tools which allow any 'ageing' phenotypes to be quickly identified and dissected. The chief disadvantage of these classic short-lived models is that they are a long way (phylogenetically speaking) from humans. To that end, there is a need for alternative short-lived ageing models that are closer to mammals (Gerhard, 2007). A particularly promising candidate is the zebrafish (*Danio rerio*). This animal is small, prolific, easily reared and has excellent molecular genetics resources available due to its use as a developmental model (forward and reverse genetics can be performed in zebrafish and there are a variety of expression constructs available which permit the construction of transgenic animals). Some physiological parameters are perturbed in the animal (e.g. circadian rhythm) that are of direct relevance to human ageing and thus potential avenues of research for groups seeking to improve quality of life for the elderly (Zhdanova *et al.*, 2008). The chief disadvantage of the animal is, ironically, that it lives rather too long. Zebrafish have a maximum lifespan of about 5–6 years (and a mean lifespan of about 3.5 years). This is

longer than that of the laboratory mouse, *Mus musculus*, which has a maximum lifespan of about 4 years. Promising alternative short-lived vertebrates are the killifish (*Nothobranchius furzeri*) and a closely related species, the Japanese medaka (*Oryzias latipes*). The medaka lives about as long as a mouse (2–4 years) but has a small genome (~800 Mb) that has already been cloned into BAC libraries. The killifish in contrast is much shorter-lived (just 3 months) and has the potential to represent a superb ageing resource. However, there is an almost complete lack of molecular or cellular tools for the study of the organism (although its genome seems to have a relatively high homology to that of medaka, which may facilitate molecular biology studies). In addition, *Nothobranchius* is potentially challenging to rear because the organism has a requirement for embryonic diapause (in the wild, killifish lay eggs in ponds which dry up during the summer). Establishing proper husbandry techniques for the animals is thus not trivial, but is likely to be rewarding for any group able to make the investment.

One other short-lived organism of great potential utility to ageing researchers is the pond snail, *Lymnaea stagnalis* (Patel *et al.*, 2006). Compared to higher organisms the snail has a relatively small central nervous system (~20 000 neurones) and has been extensively studied with regard to learning and memory (it can display both appetitive and aversive conditioning response) and as a result the neural circuits underlying a variety of physiological responses (including rhythmic feeding and respiration) are well understood. Recent studies have extended this analysis to the neurobiology of ageing in the animal and have found that the feeding response is significantly blunted with age (old animals bite less frequently following a stimulus and take longer to swallow food). This 'senility' is driven by changes in the connectivity of a pair of serotonergic neurones known as the cerebral giant cells (CGCs). Thus, although *Lymnaea* currently lacks a sequenced genome this is compensated for by its short lifespan (typically around 10–12 months) and the wealth of data available on cognition in the young animal.

### 4.2 *Long-lived ageing models*

If the advantage of short-lived organisms to ageing research is speed then the advantage of ultra-long-lived organisms is staying power; or more precisely, the fact that the organism in question is able to maintain normal physiological function for a much longer period than is possible for a human. Organisms which are documented as having much longer lifespans than humans are actually quite rare, and gerontologists seeking to work with them require some way of actually dating the organism to see how old it is (typically through some form of annual growth ring). Creatures known to live longer than humans include rockfish (~200 years), bowhead whales (at least one specimen dated to over 200) and sturgeon (~150 years of age). However, none of these organisms is suitable as a laboratory species for reasons which do not require elaboration (*Balaena mysticetus* weighs ~50 000 kg and is about 15 m long). The best candidate for an ultra-long-lived laboratory animal is almost certainly *Arctica icelandica* (the quahog or cyprine), a suspension-feeding bivalve mollusc which lives in deep water (below 25 m) in the shelf seas off the North European and North American continents. The species is dioecious (separate sexes), with larval development taking between 30 to 60 days and individuals become sexually mature within 15 years. Crucially, *Arctica* shells contain a growth record of the animal in the form of wide annual summer growth increments separated by narrow winter growth lines. This allows the organisms