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Review

Flies, worms and the Free Radical Theory of ageing

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ABSTRACT

Drosophila and *Caenorhabditis elegans* have provided the largest body of evidence addressing the Free Radical Theory of ageing, however the evidence has not been unequivocally supportive. Oxidative damage to DNA is probably not a major contributor, damage to lipids is assuming greater importance and damage to proteins probably the source of pathology. On balance the evidence does not support a primary role of oxidative damage in ageing in *C. elegans*, perhaps because of its particular energy metabolic and stress resistance profile. Evidence is more numerous, varied and consistent and hence more compelling for *Drosophila*, although not conclusive. However there is good evidence for a role of oxidative damage in later life pathology. Future work should: 1/ make more use of protein oxidative damage measurements; 2/ use inducible transgenic systems or pharmacotherapy to ensure genetic equivalence of controls and avoid confounding effects during development; 3/ to try to delay ageing, target interventions which reduce and/or repair protein oxidative damage.

1. Introduction

The ground has recently shifted under the Free Radical Theory of ageing. There have been tremors for the past two decades and warnings sounded, but the degree to which these were heard and then further investigated has only recently increased to the extent that it is beginning to be heard by the mainstream.

The report of the proceedings of the Dahlem Workshop on molecular aspects of ageing, held in Berlin 1994 (Esser and Martin, 1995), was remarkable for one thing in particular. Amongst several chapters documenting evidence supporting the Free Radical Theory of ageing, Swartz and Maeder (1995) concluded that "The free radical theory of ageing . . . is not well supported by existing data" and that ". . . the evidence for oxidative damage being the principal cause of ageing is not strong and unambiguous".

In the same report, Sohal and Orr (1995) stated that "The most direct and probably the strongest supportive evidence (for oxidative stress causative of ageing) is that overexpression of Cu–Zn superoxide dismutase (SOD) and catalase genes increases the average and maximum lifespans of *Drosophila melanogaster* by up to one third and delays the age-related loss of function". Orr and Sohal (2003) presented data refuting the conclusions of their earlier work (Orr and Sohal, 1994).

However the Free Radical Theory of ageing was and is still accepted unquestioningly by many, especially in biomedical research. Whether it turns out to be true or not, such uncritical attitudes can be, and probably have been, detrimental to research.

Here we review the vital role played in the development of this field by studies using invertebrate model organisms, including their advantages, limitations and potential for future work. As well as covering work which has been most influential, we have tried also to include work which we think should have been, and should be, more influential. As has become almost inevitable in biology, this work has uncovered a daunting complexity beneath what began as attractive simplicity.

2. Free Radical Theory – early history

Connecting observations from comparative physiology (metabolic 'rate of living' theory) with radiation biology (oxyradical generation), Denham Harman proposed that functional decline in cells and tissues was due to the cumulative effects of macromolecular damage caused by oxygen radicals produced by respiratory enzymes (Harman, 1956). This was the Free Radical Theory of ageing.

It is an extraordinarily attractive theory. The mechanism of reactive oxygen species (ROS) production is universal among animals, as is the typical Gompertzian trajectory of population mortality. But most of all, it is a mechanism that causes damage as a by-product of normal living, damage which accumulates over time (Stadtman and Levine, 2000).

The links between normal ROS production, damage and ultimate effects on the organism are not conclusive. The main reason why such a link may be obscure is represented by much of the work that has occurred in the years since Harman's paper; that the steps from

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ROS production to organism health are many, often interlinked and involved in feedback systems, and admit a huge degree of variation, both environmental and genetic. This variation reflects, perhaps causes, the variation we see in lifespan within and between taxa.

3. ROS production

One- and two electron reductions of oxygen produce superoxide and peroxide respectively, largely at complexes I and III of the electron transport chain in the mitochondria, and can be produced at a rate of 1–4% of each oxygen molecule used (Brand et al., 2004; Kell, 2009). Typically production is highest when oxygen, the terminal electron acceptor, is limiting (e.g. hypoxia, anoxia, ischaemia), or when substrates are in excess. There are many immediate mechanisms to cope with these potentially damaging byproducts, inherited in common from ancient bacterial ancestors (Imlay, 2008), and each has, to a greater or lesser extent, been the subject of manipulation in invertebrate model organisms with the aim of reducing oxidative damage and extending lifespan.

3.1. The role of iron

Biochemical work showed that doses of superoxide or peroxide alone needed to damage biomolecules in vitro were far too high to be physiologically relevant, implicating other ROS species (reviewed in Imlay, 2008). In an extensive review of the possible role of poorly-liganded iron in ROS production, disease and ageing, Kell (2009) notes that the most damaging ROS species, the hydroxyl radical, is produced largely by conversion of peroxide by exposure to free or incompletely liganded Fe ions. In normal metabolism, the Fenton reaction of peroxide with unincorporated ferrous ion bound to lipids and DNA and bound to or constituent of proteins (iron–sulfur clusters) is probably the main source of oxidative damage to these macromolecules.

Iron accumulates with age in *Drosophila* (Massie, 1984; Massie et al., 1985) and other species, including mammals (Massie et al., 1983). In fact, feeding flies with tea extracts reduced iron accumulation with age and was associated with lifespan extension of up to 21% (Massie et al., 1993). Investigating the effects on lifespan of more specific iron chelating agents, especially later in life, should shed light on this potentially important effect.

3.2. The relationship between ROS production and ageing

ROS production increases with age in mammals (e.g. Nabben et al., 2008) and in flies. Peroxide production doubled in mitochondria isolated from older houseflies, as did activities of a range of respiratory enzymes. Interestingly also, oxidative damage imposed in vitro to the isolated aged mitochondria led to an increased peroxide production (Sohal and Sohal, 1991). Similarly, peroxide production increases with age in *Drosophila*, but while both diet restriction (DR) and inactivity decrease oxidative damage (and increase lifespan), DR has no effect on, and inactivity increases, peroxide production (Cocheme et al., 2011) suggesting a significant potential disconnect between ROS production and oxidative damage.

Perhaps this is unsurprising. Fig. 1 shows a schema describing the process from ROS production to organism ageing, and lists, non-exhaustively, the large range of factors which can influence the steps of this process. Each one of these factors is subject to genetic and/or environmental influences.

The lifespan extension due to DR may be unrelated to ROS production in *Drosophila*. Although whole body mitochondrial extracts necessarily overrepresent flight muscle, lifespan increase with DR was not associated with reduced ROS (Partridge et al., 2005), and reducing ROS production by genetically lowering membrane potential failed to increase lifespan (Miwa et al., 2004). However in rats DR reduces membrane potential and does reduce peroxide production from isolated mitochondria (Ash and Merry, 2011), raising the issue of comparability of invertebrates to mammals in this area.

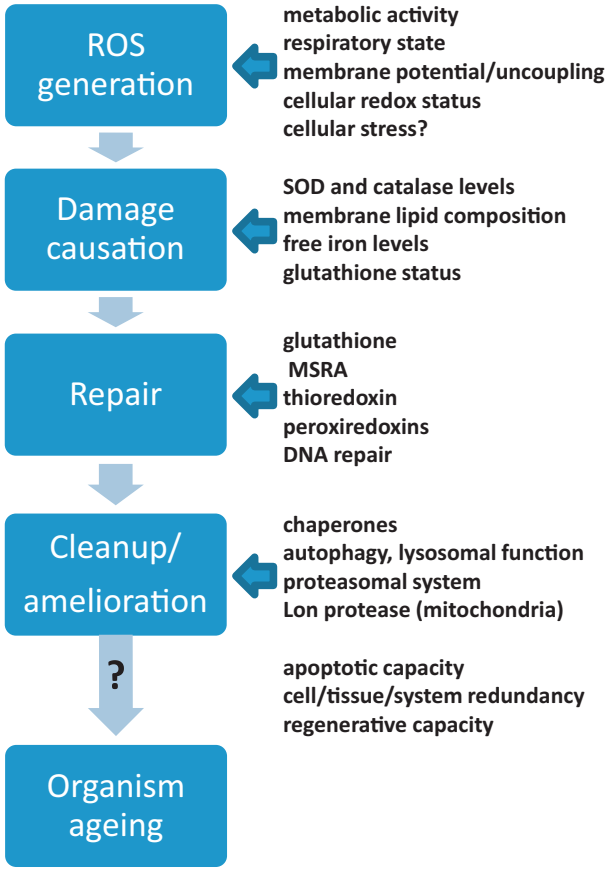

Fig. 1. Schema for stages of the process from ROS production to effects on organism health, with (non-exhaustive) lists of potential modulators at each stage.

Indeed, in the nematode worm *Caenorhabditis elegans*, metabolism is very different. They can tolerate very high oxygen tensions and are capable of significant anaerobic metabolism. Suppressing almost every respiratory chain gene during development (but not adulthood) significantly extends lifespan, possibly by upregulating non-aerobic metabolism and reducing superoxide production, although this is still a hypothesis (reviewed in Muller et al., 2007).

4. Oxidative damage: types, measurement, and ageing

The routes and reactions by which ROS damage lipids, proteins and DNA are well covered elsewhere (Halliwell and Gutteridge, 2007). We will discuss the common end-products, their measurement and use in ageing research.

Early work aimed to demonstrate that oxidative damage increases with age, by comparing groups with ostensibly different physiological ages. Physiological age refers to the amount of its ultimate lifespan an organism has lived. Typically this has been achieved by assaying young vs. older animals, or by using a measurable proxy of physiological age, or imposing dietary restriction or other methods to impose differential rates of mortality. Invertebrates have been especially useful for this.

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4.1. Lipid oxidation

The most commonly measured markers of lipid oxidation are the reactive aldehydes 4-hydroxynonenal (HNE) and malondialdehyde (MDA). Their half lives are in minutes (c.f. microseconds for ROS) although they are reactive so tend to indicate processes ('oxidative stress') occurring at the time of sampling, rather than being markers of accumulated damage. However among their detectable end-products which can accumulate are adducts of proteins (lysine residues) and DNA, which is mutagenic (Hulbert et al., 2007). HNE and MDA are relatively easily detected and commercial kits are available; lipoxidatively modified protein requires antibody-based assays and modified DNA detection is technically complex and rarely done.

Lipid oxidation as a cause of ageing rather than as an indicator of oxidative damage has received relatively little attention however the work of Hulbert (see Hulbert et al., 2007), mostly in mammals, has sparked interest.

Among mammals, maximum life span potential is inversely correlated with membrane peroxidizability, which is a function of membrane phospholipid profile. Long chain polyunsaturated fatty acids (PUFAs) are many times easier to peroxidize that are saturates, monounsaturates and di-unsaturates.

Ectotherms often have relatively low levels of unsaturation in their membranes; *Drosophila* have no long chain fatty acids (>C18), C18:3 is generally at <5% and C18:2 less than 20%, whereas mammalian membranes commonly contain substantial quantities of 20:4, 20:5 and 22:6 fatty acids, making their membranes around two orders of magnitude more peroxidizable than those of *Drosophila*. For this reason (Shen et al., 2010) argue that the fly is not a valid model for studying lipid metabolism and disease, but this does not preclude research into the basic mechanisms of ageing, and studies of other invertebrates have been very informative in an ageing context.

Protein lipoperoxidation end products were assayed in *Drosophila* under different temperature regimes, and the changes in their levels scaled with the % mortality of the fly cohorts (Zheng et al., 2005), although there was no increase in any case until about 20% of flies had died. If oxidative damage occurs substantially only later in life, more as a cause than as a consequence of ageing, this result admits the possibility that later observed increases might be due to heterogeneity of physiological age within the samples of 20–30 flies, such that samples may contain more flies close to death and hence carrying much more lipoxidatively modified proteins. This is a difficult problem with flies and worms, and will be explored further. To further complicate, when measured under diet restriction there was no difference in damage trajectories between low and medium calorie food despite substantial differences in survival.

C. elegans, unusually for an ectotherm, can synthesize and contains long chain PUFAs. Two lipid properties were correlated with lifespan across a range of mutant strains: chain length and susceptibility to oxidation. Shmookler-Reis et al. (2011) suggests that greater longevity across the strains is associated with reduced unsaturation, and the consequently increased membrane fluidity is compensated by increased chain length. This longevity finding was largely supported by RNAi experiments in wild type worms to knock down elongase and desaturase genes. Dietary lipids also produced lifespan effects in the expected direction.

Dietary effects on lipoperoxidation are not confined to *C. elegans*. In honeybees the difference in membrane peroxidizability between (genetically identical) queens and workers is large enough to explain the difference in their longevity (Haddad et al., 2007), and lipoperoxidation of lymphocyte DNA in humans was increased by diets high in PUFAs (Fang et al., 1996).

4.2. Oxidative damage of DNA

DNA is subject to mutation as a result of oxidative damage, including single and double strand breaks, and adducts of bases and sugar groups. The most commonly measured adduct is 8-oxo-2,7-dihydro-2′-deoxyguanosine (oxo8dG). It is strongly mutagenic, causing increased GC-TA transversions so may have little effect in postmitotic tissues. This combined with technically difficult measurement and the lack of a lifespan effect in mice deficient for its repair enzyme (Osterod et al., 2001) has resulted in a preference for measuring other types of DNA damage.

The somatic mutation theory holds that ageing is due to accumulation of DNA damage over time which leads eventually to dyshomeostasis, disease and death. While there is no question that DNA damage is required for the development of neoplastic diseases, there has been less work on the role in ageing of oxidative damage to DNA, for three main reasons. Firstly, the damage has been difficult, expensive, and unreliable to measure. Secondly, damage on one chromosome may not lead to dramatic dysfunction so long as the homologue is undamaged at the same locus.

But mostly the relative lack of interest, at least using invertebrates, comes from early work on haplodiploid wasps. Clark and Cole (1967) reviewed and extended a body of work whose central hypothesis was: if DNA damage causes ageing, there should exist major differences in lifespan between ploidy types within a species. This is based on the idea that damage to a gene which exists as a single copy in a cell should be more harmful ultimately to the organism than damage where a second (undamaged) copy exists. The fact that such lifespan differences were not seen remains a powerful argument against somatic mutation in nuclear DNA being a significant causative player in ageing.

More recent evidence comes from *Drosophila*; DR extended lifespan but did not alter the accumulation of spontaneous mutations with age, measured using an incorporated lac-Z plasmid construct (Edman et al., 2009). Although whole fly homogenates were used to measure the mutations, the authors did not feel that the study lacked power to detect mutations in specific cell types, so long as the tissues were not too small, and unpublished results using head, abdomen and thorax separately showed that accumulation of mutations with age was greatest in the thorax. However the authors do note the differences in DR administration between flies and mice, and the possibility that lifespan extension may occur by different means in the two species.

4.2.1. mtDNA mutation rate in flies and worms

While potentially mutagenic DNA damage might be consequence-free in postmitotic nuclei, the case is different for mitochondrial DNA (mtDNA), whose replication is independent of mitosis. A mitochondrial focus for the free radical theory has been especially attractive because of the assumed vicious cycle, i.e. ROS cause mtDNA mutations, mutated electron transport chain proteins malfunction, ROS are overproduced.

The mechanism is supported (but by no means proven) by observations of respiratory chain complex II-defective *C. elegans* (*mev-1* mutants) which show shortened lifespan. But it is very easy to shorten lifespan by mutating genes; it does not indicate that the gene is involved in ageing. Indeed Copeland et al. (2009) reduced expression of a range of mitochondrial genes using RNAi in adult *Drosophila* and generally observed increased lifespan.

However measures of actual mtDNA mutation tend to be quite low. Large deletions, point mutations and tandem duplications appear typically in less than 1% of mtDNA from ageing human tissues (reviewed in Wei and Lee, 2002). Tissue specific deletions have been detected differentially in old flies (Yui et al., 2003), associated with flanking repeats, but quantitation was not possible. And while increasing mtDNA mutation by interfering with proofreading

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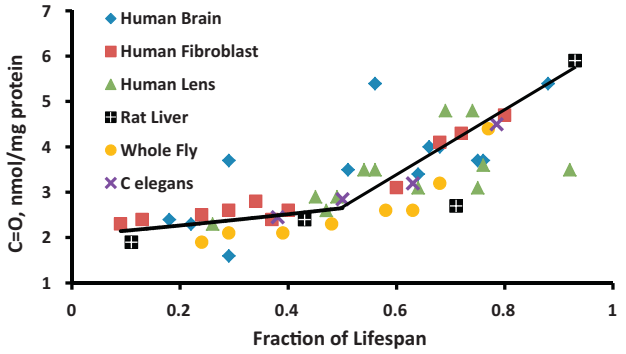

Fig. 2. Relationship of protein carbonylation with physiological age, taken from Levine (2002).

in mice reduces lifespan with some phenotypes resembling ageing (Trifunovic et al., 2005), it does not increase ROS production, and another method of increasing mtDNA deletions produced no premature ageing phenotype (Tyynismaa et al., 2005).

Based on substantial data to date, accumulation of somatic nuclear and mtDNA mutations with age are likely to be more a consequence than a cause of ageing.

4.3. Protein oxidation

Protein carbonylation is by far the most frequently measured of all indices of oxidative damage, mostly using techniques based on the derivatization protocol of Stadtman, Levine and colleagues (Levine et al., 1990). Their work, and that of many others, makes a powerful case for the importance of this protein oxidation in ageing; they estimate that, toward the end of life at least one in three proteins is carbonylated (Stadtman and Levine, 2000). They note that this level of modification must surely affect biological function. Interestingly, the measurement methods employed also estimate that one in ten proteins are carbonylated in young animals.

Fig. 2 is reproduced from Levine (2002) using data referenced therein, showing protein carbonyl levels as a function of fraction of lifespan elapsed. An exponential line is the best single linear fit but the two stage line used by Levine fits better. Although there are difficulties accurately gauging 'fraction of lifespan' in flies, worms and rat liver due to the necessary destruction of the animals, the data does suggest a slow increase up to about half way through life, followed by a much more rapid increase in later life.

Carbonyl content correlated with chronological age (whole body homogenates and mitochondria) and with a measure of physiological age in houseflies (whole body homogenates – "crawlers" vs. "fliers") in both (Sohal et al., 1993). Rates of ROS production and levels of protein carbonylation were correlated with maximum lifespan potential across five species of flies (Sohal et al., 1995). Preventing flight activity extends housefly lifespan and reduces age-related protein oxidative damage (Yan and Sohal, 2000).

Protein oxidation with age seems to vary across tissues, at least in species where such measurements are possible. Unfortunately, measuring tissue-specific protein oxidation is difficult in flies and practically impossible in *C. elegans* so informative work has largely been in mammals. Susceptibility to X-ray induced oxidative damage of tissue homogenates correlated with maximum lifespan potential across mouse, rabbit, pig, rat and pigeon; older animals more susceptible than young and brain was more susceptible than heart tissue (Agarwal and Sohal, 1996). Montine et al. (2002) found no increase in carbonyl levels in cerebral cortex of 26-month-old mice. Conversely, total plasma carbonyl content increased with age in rats but not mice, but increased carbonylation with age of different specific plasma proteins was observed across mice, rats and rhesus monkeys (Jana et al., 2002). This interesting result indicates that non-specific carbonylation of plasma proteins, at least, is not responsible for ageing.

ROS damage some proteins much more than others. In housefly ageing, two mitochondrial proteins were carbonylated substantially more than others. Aconitase, as part of the citric acid cycle, if reduced may block electron flow to oxygen leading to accumulation of reduced NADH, increasing rates of oxidation. Adenine nucleotide translocator (ANT) has (at least) a dual function. As well as ADP/ATP exchange, it functions as an uncoupler of respiration by reducing membrane potential, to which peroxide production is proportional. Mild uncoupling has extended lifespan in mice (da Silva et al., 2008), and is seen under DR conditions in rats (Ash and Merry, 2011). Particular carbonylation of these two proteins provides the possibility of a positive feedback system in terms of ROS production by mitochondria with age (Levine, 2002).

The volume of supportive correlative data is large, although often with an effective sample size of only two (e.g. DR vs ad-lib fed, young vs old, treated vs control). But they do not, still, distinguish between causation and correlation. Therefore exceptions, whether they be individual animals or taxa, are especially instructive. One such is the naked mole rat. This exceptionally long-lived animal carries high basal levels of oxidatively modified proteins, lipids and DNA (Andziak et al., 2006), in contrast to comparisons between species where maximum life span potential correlates inversely with levels of protein carbonylation. The naked mole rat shows lower antioxidant defences but higher protein stability with age, associated with increased proteasome activity, suggesting the importance of maintenance and repair mechanisms in this animal (Perez et al., 2009).

Unfortunately the great majority of intervention studies fail to measure oxidative damage, making clear support (or otherwise) for the role of ROS in ageing impossible.

4.3.1. Tyrosine nitration

Superoxide can react endogenously with nitric oxide to produce the peroxynitrite radical. Nearly as reactive as the peroxyl radical, this species can nitrate cysteine, methionine, tryptophan and most importantly tyrosine, where the modification prevents phosphorylation. This can prevent the actions of kinases, potentially disrupting the multiplicity of signal transduction networks which their actions mediate. This mechanism, under the influence of increased cellular oxidant release, may have been responsible for insulin receptor nitration and inactivation, and associated glucose intolerance observed following long term intermittent feeding in rats (Cerqueira et al., 2011). Protein nitration is a relatively new area of study; reliable detection and quantitation is beginning to become more mainstream (Schoneich and Sharov, 2006), and its role in diseases of ageing, if not ageing itself, is being steadily documented.

4.3.2. Lipofuscin

Examining the accumulation of lipofuscin in postmitotic cells illustrates well the major limitation of these types of correlative study; is oxidative damage a cause or a consequence of ageing? Lipofuscin has long been regarded as a biomarker of ageing, although in flies it is difficult to use this term as it is usually reserved for parameters that can predict life-span or longevity. Unfortunately in flies, this is not the case as they must be sacrificed for the assays. Jacobson et al. (2010) showed a correlation between lipofuscin accumulation and lifespan in *Drosophila* across life-span altering interventions. Lipofuscin is a high molecular weight fluorescent heterogeneous material composed mainly of lipid and protein residues which have cross-linked (Chowdhury et al., 2004).

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Although the process of its production, eventual deposit in secondary lysosomes and accumulation with age is unclear, lipid peroxidation is involved (Chowdhury et al., 2004; Schutt et al., 2003; Yin, 1996). It might be that lipofuscin is never totally eliminated and so builds up due to constitutive lipid peroxidation over time, thus a direct result of oxidative damage and possibly a cause of ageing. Or it might increase due to any or all of: age-related decline in lysosomal activity, increased autophagocytosis, or decreased exocytosis (Terman and Brunk, 1998), from whatever causes. As pointed out by Beckman and Ames (1998), since lysosomal proteinase inhibitors administered to rats and dogs caused rapid accumulation of lipofuscin-like granules (Ivy et al., 1989) and lysosomal activity decreases with age (Rajawat et al., 2009), lipofuscinogenesis may be a consequence rather than a cause of ageing.

This area almost certainly suffers, or at least has suffered, from significant publication bias, such that negative or unsupportive results have remained unpublished. However the weight of evidence is considerable for oxidative damage, especially to proteins, as a major cause of increasing chance of mortality with time, especially perhaps in older age. To conclude the section on ROS-mediated protein damage, Stadtman notes that "...although the scientific literature abounds with positive correlations between free radical-provoked damage and ageing, proof that such damage is a cause of ageing is still lacking." (Stadtman, 2002). This admirable note of caution might be balanced by a question: what would such proof look like, and is it possible to obtain?

5. Interventions

Because we do not understand the tempo and mode of ageing – the causes of increased risk of mortality with chronological age – it is difficult to place much weight on studies in which interventions shorted lifespan and generally they will not be discussed. They include antioxidant gene knockouts and experiments involving increased ROS generation using hyperoxia or paraquat.

5.1. Genetic manipulations

One of the most influential pieces of evidence for the Free Radical Theory of ageing was the lifespan extension seen in transgenic lines of *Drosophila* which overexpress superoxide dismutase (SOD – which converts superoxide to peroxide) and catalase (which reduces peroxide to water and oxygen) (Orr and Sohal, 1994). This paper has been cited over 920 times. Critical examination and extension of their own and others' work in this area by Orr and coworkers (Kaiser et al., 1997; Orr et al., 2003; Orr and Sohal, 2003; Tatar, 1999; Tower, 1996) concluded that the lifespan extension was only seen when controls were short-lived,. They then produced comprehensive data using relatively long-lived controls and showed no lifespan extension associated with overexpression of a range and combinations of antioxidative enzymes (Orr et al., 2003). This paper has been cited 90 times but since 2004, the original, whose conclusions it corrects, has been cited more than 300 times. A brief examination of these citing papers confirms that excepting very recent works, the great majority used the original study as broad support for a role of ROS in ageing. Taking an egregious example, in their 2011 review of the SOD enzyme (D'Alessandro and Zolla, 2011) cite the 1994 paper but not the 2003.

Despite caution having been voiced about methodology in general and the dataset in particular (e.g. Kaiser et al., 1997; Tatar, 1999; Tower, 1996), the critical importance of genetic background in the context of transgenic studies is, thankfully, a point being made much more emphatically now with empirical work (e.g. Toivonen et al., 2007). Overexpressing human SOD1 in *Drosophila* motoneurons extended mean lifespan by 40% (Parkes et al., 1998), however when this experiment was repeated by placing the transgenic construct on ten different long lived wild type genetic backgrounds, significant although quite modest lifespan extension was seen on six of ten genotypes in females and only one in males, achieving nothing like the 40% increase in mean lifespan seen in the original experiment (Spencer et al., 2003).

Genetic background is important for two reasons. Firstly, the controls should be genetically relevant. In *Drosophila*, the GAL4-UAS system for overexpressing genes uses two constructs – one which drives expression of the other. The controls, typically, are the parental strains each of which contain one of the two required constructs and typically are genetically relatively unfit compared to wildtype, and typically have not been outcrossed to wild type. Therefore the offspring containing both constructs usually has a genetic background considerably more heterozygous than the controls and it is not possible to ascribe a lifespan effect, which is a fitness increase, to the transgene because heterosis (hybrid vigour) cannot be excluded as a cause. A clue to instances where heterosis is a cause is that typically control survival curves have significant age-independent, or baseline, mortality, sometimes referred to as frailty.

Genetic background is important also because increased ROS detoxification may actually ameliorate some of the pathologies caused by frailty encoded by a short-lived genetic background. Indeed it appears that these ROS-related genetic and pharmacological interventions can extend lifespan in (short-lived) models of some age-related diseases. This will be examined later.

Despite these caveats, some results remain compelling. In particular, overexpression of methionine sulfoxide reductase (Cirelli, 2006), and overexpression of glutamate-cysteine ligase (Orr et al., 2005) in *Drosophila* have shown substantial lifespan increases.

Methionine sulfoxide reductase A (MSRA) reduces oxidized methionine residues. Overexpressing MSRA neuronally extended median lifespan by 70% on average versus long lived GAL4-UAS driver and responder lines, and when globally overexpressed, median lifespan was about 40% longer than the longest lived control. In addition, activity levels and reproductivity indices were delayed in their decline in overexpressing flies. The MSRA result is probably the most compelling of all transgenic studies in *Drosophila*.

Glutamate cysteine ligase (GCL) is the rate limiting enzyme in glutathione biosynthesis. When overexpressed neuronally, the catalytic subunit of GCL extended mean and maximum lifespan by up to 50% versus long-lived GAL4-UAS driver and responder lines. When the modulatory subunit was overexpressed globally the increase was up to 24%.

However several issues remain unresolved. In both of these studies the GAL4-UAS system was used, which overexpresses the target throughout development and adulthood. Ideally we would exclude effects exerted during development. There remains the fact that oxidative damage was not measured. With regard to the issue of genetic equivalence of controls, potentially important is the lifespan extension seen when MSRA was overexpressed predominantly in the eye, which would not be expected to extend lifespan. This provides a suggestion of the contribution of heterosis to the lifespan extension observed, which was significant but not large. The fact remains that no-one else has achieved a lifespan of 120 days at 25C, but until the results are validated using more genetically equivalent controls and during adulthood only, the result cannot be taken as totally conclusive.

The situation in *C. elegans* overall muddies, rather than clarifies, because of the very large number of genes, over 300 now (Johnson, 2008), whose knockout or knockdown seems to extend lifespan. However one theme is discernible: many 'gerontogenes' in *C. elegans* increase resistance to stressors, including oxidative stress (Johnson et al., 2001). To take a well known example, the

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long-lived daf-2 mutant displays reduced protein carbonylation despite mitochondria producing more peroxide, especially in older animals, but SOD, catalase and glutathione are all upregulated (Brys et al., 2007). Many of these genes activate elements of the dauer pathway, a non-feeding, immobile, non-reproductive and highly stress resistant larval state entered in response to low nutrition or crowding, during which ageing practically stops. The dauer state highlights the ecology of *C. elegans* which, as with all model organism research, demands a cautious approach when extrapolating results to humans.

Some ROS-specific studies deserve mention. Lifespan extension with Sod-2 overexpression is not caused by reduced oxidative damage (Cabreiro et al., 2011). Similarly but conversely, Sod-2 deletion extended lifespan but caused increased oxidative damage (Van Raamsdonk and Hekimi, 2009); a lifespan extension associated with increased oxidative damage might argue for a limited role of ROS damage in *C. elegans* ageing. To try to rescue a role for ROS damage in *C. elegans* ageing we might invoke the possibility that, in these studies, other stress resistance mechanisms became activated. The concept of hormesis will be discussed in Section 6.

As a postscript to this section, comparative enzymology suggests that modulating expression of these enzymes may provide some benefit to lifespan in mammals, but not generally. Correlating lifespan with five antioxidant activities in three tissues across 14 mammalian and avian species, the only significant results were for MnSOD and for catalase, both in brain tissue only. Glutathione peroxidase, reductase and Cu/ZnSOD activities showed no correlations with maximum life span potential (MLSP) (Page et al., 2010). Remarkably this result broadly reflects the relevant body of literature using transgenic *Drosophila*, and raises the question of the importance of tissue specificity when considering pharmacological intervention; one tissue may be helped while another is harmed.

5.1.1. Inducible or adulthood-only transgenics

While it is possible, indeed likely, that gene action during development will modulate adult ageing, generally we wish to understand the process of adult ageing and how it might be modulated during adulthood. Therefore it is important to separate developmental from adult genetic effects of interventions on lifespan. This is why inducible genetic systems and pharmacological interventions should be used much more than they currently are. For example, genome-wide screens using RNAi for lifespan increase identified about 100 genes from a range of ontological groups in *C. elegans* (Hamilton et al., 2005; Hansen et al., 2005). A screen using RNAi constructs vs. vector controls in post-development worms identified 64 lifespan extending genes from 2700 tested (Curran and Ruvkun, 2007). This represented a success rate of 2.4% c.f. 0.6% overall for the previous studies, and was almost certainly due largely to avoidance of larval stage lethality and developmental arrest. If a potential anti-ageing target gene has negative effects during development but positive during adulthood, a temporally constitutive genetic effect, whether it be overexpression by GAL4-UAS in *Drosophila* or null or hypomorphic mutation, could hide an important finding.

Inducible systems also use genetically identical controls, which is a critical advantage.

Noting the particular suitability of inducible systems to lifespan studies (Sun and Tower, 1999; Tower, 2000), the Tower lab has published numerously using inducible transgenic systems in *Drosophila*, revisiting antioxidant gene overexpression. Overexpressing MnSOD and Cu/ZnSOD (but not catalase) extended lifespan using the inducible FLP-out system in young adults (Sun et al., 2002; Sun and Tower, 1999). The (small) caveat here is that two heat shocks were used to achieve the overexpression, which is likely to have a positive effect on lifespan, however for MnSOD the lifespan extension was proportional to the degree of overexpression observed. Later work showed reproducible lifespan extension using GeneSwitch, in which the GAL4-UAS system is activated only when the flies are fed a drug, and similarly the Tet/on system (Curtis et al., 2007; Ford et al., 2007). Thus it is possible to accurately time the effect of the transgene, whether overexpressing or RNAi-mediated knockdown, and dose can be varied to some degree.

Geneswitch RNAi-mediated knockdown in adult flies of mitochondrial respiratory complex I globally and several complexes neuronally leads to increased mean life span. The mechanism is unclear; increases in lifespan were not always reflected by increased resistance to oxidative stress induced by paraquat (Copeland et al., 2009), and oxidative damage was not measured.

5.2. Pharmacological interventions

In theory worms and flies are excellent platforms for drug screening and testing, indeed worms are used on industrial scales by some companies, however the picture from experimental studies is unclear with respect to the free radical theory. Platinum nanoparticles (SOD/catalase mimetic) extend worm lifespan by 22%, associated with the expected changes in an array of ROS-related parameters (Kim et al., 2008). However the salen manganese SOD mimetics EUK-8 and EUK-134 did not repeatably extend LS in worms, despite large increases in mitochondrial SOD activity and increased survival on paraquat, nor in *Drosophila* nor in houseflies (reviewed in Gems and Doonan, 2008). In *Drosophila*, like in mev-1 worms, they could ameliorate negative effects of compromised defences (Magwere et al., 2006; Melov et al., 2000). Finally, testing six plant-derived antioxidants in worms, in vitro activity did not predict in vivo efficacy, and did not predict lifespan extension (Pun et al., 2010); lifespan extensions observed were not due to direct antioxidant mechanisms.

There is otherwise very little of relevance published in *Drosophila* involving the testing of ROS-relevant compounds for effects on ageing. It is to be hoped that this is not due to non-publication of reliable negative results.

In humans, meta-analysis of randomized controlled trials showed that selenium and vitamin C have no effect while standard antioxidant supplementation (vitamins A and E and beta-carotene) actually increases mortality.

6. Hormesis – good radicals and bad radicals

The concept of hormesis in ageing – the idea that a seemingly detrimental stimulus can provide a fitness advantage later (i.e. longevity) was in the wilderness for a while, due to lack of an obvious mechanism, but ROS may now be providing. Reducing glucose metabolism in *C. elegans* led to overproduction of superoxide, upregulated oxidative stress resistance and extended lifespan (Schulz et al., 2007), and provided the first real empirical support for the concept of mitohormesis (hormesis mediated by mitochondrial metabolism). Regarding Kim Zarse and Michael Ristow's subsequent work in humans, which showed that antioxidants abrogate the exercise-induced gains in insulin sensitivity and ROS defence, one is perhaps unsurprised at the failure of SOD overexpression to extend lifespan, since superoxide or a proximal downstream product is likely to be the hormetin here.

One possible candidate is the lipoxidation product HNE, which has important signalling functions; fat accumulation is triggered by HNE in mice and in worms, suggesting a broad conservation of the mechanism (Singh et al., 2009). Superoxide plus free fatty acids promote mitochondrial uncoupling in vitro, likely through the production and direct action of HNE on uncoupling proteins (Esteves et al., 2006), which reduces superoxide production by using energy to produce heat (Brand, 2000).

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7. Oxidative damage and protection in genetic models of disease

Salmon et al. (2010) make a persuasive case: ROS damage may be an important determinant of lifespan under stressful conditions, stress which may be endogenous or exogenous. In most studies of model organisms, there is little or no exogenous stress (except perhaps dietary – overfeeding), a state which poorly simulates nature. However endogenous stress may be caused by disease states, explaining the involvement of ROS damage in many diseases of ageing, although the question and argument becomes circular; what caused the disease states or susceptibility to them?

While a primary role for ROS in causing ageing may not have unambiguous support, a role in age-related diseases is often well supported empirically, including type 2 diabetes, atherosclerosis and diseases of protein aggregation including Alzheimer's Disease, Parkinson's Disease and Lewy Body Dementia. This role has been investigated using invertebrate models of human neurodegenerative diseases.

In Parkin mutants, a short-lived fly model of Parkinson's disease, overexpression of SOD1 and supplementation with metal chelators and antioxidants each prolonged lifespan substantially (Saini et al., 2010). Similarly, in another *Drosophila* Parkinson's model, alpha-synuclein-induced loss of dopaminergic neurons was suppressed and (short) lifespan extended by overexpressing Nrf2 and/or knocking down Keap-1 (Barone et al., 2011), and by overexpressing SOD1 (Botella et al., 2008).

Sniffer is a carbonyl reductase gene in *Drosophila*. When inhibited it resulted in neuronal death and shortened lifespan, and when overexpressed it retarded age-related locomotor decline suggesting a neuroprotective role via reducing levels of oxidative damage (Botella et al., 2004), though this did not extend lifespan.

Nrf2 is a regulator of cellular antioxidant response and phase II detoxification enzymes, conserved from flies to humans. Evidence for a broad protective function, including neurodegenerative disorders, inflammatory disorders, atherosclerosis and insulin resistance as well as it being a possible target for cancer chemotherapy, is summarized by Sykiotis and Bohmann (2010), who have investigated the gene's function using *Drosophila*. Heterozygous nulls for the negative regulator of cncC extended lifespan by a small but significant amount in *Drosophila* (Sykiotis and Bohmann, 2008), prompting the question as to whether cncC modulation during adulthood only could provide greater longevity.

We believe that Nrf2 is an example of a gene very worthy of investigation as a target for therapies to extend healthy lifespan; it activates a range of downstream damage resolution processes and does not seem to upregulate defences proximal to oxidant production, which may be detrimental to health. Nrf2 is currently a therapeutic target and at least one drug is in phase 3 trials to treat chronic kidney disease of diabetic origin.

8. Flies and worms: advantages and limitations

Apart from the general advantages of *Drosophila* and *C. elegans* as model systems (large numbers, short lifespan, inexpensive), there are some which are especially advantageous when studying ageing. In worms RNAi is generally cheap and effective and can be applied in precisely timed ways, and in *Drosophila* we can time and target gene overexpression and knockdown, and even achieve something like a dose effect, relatively easily.

However we should not be blind to the limitations of these two models. Comparing with humans *Drosophila* has more homologous genetic systems than *C. elegans* but there are still major differences and we must be mindful of the importance of ecology in shaping survival mechanisms when using these models for human ageing. For assays of oxidative damage, many individuals must be used, imposing the problem of age heterogeneity within cohorts, and it is difficult or impossible to look at different tissues. Also, because they are mostly post-mitotic we cannot usefully study regenerative capacity using these animal models. Finally, in *C. elegans* the use of N2 as a control strain for fitness assays can be problematic due to the potential for genetic non-equivalence between mutants and controls (Gems and Riddle, 2000).

9. Conclusions and questions

From this review we conclude the following:

- *C. elegans* has very different energy metabolism from *Drosophila* (and mammals), combined with particularly robust stress response mechanisms associated with the dauer pathway, so results should be interpreted with that in mind.
- Wherever possible, inducible transgenic systems should be used, or other methods whereby the intervention is applied only during adulthood.
- Oxidative damage to DNA probably does not contribute significantly to ageing.
- If oxidative damage is not involved in causing ageing (but it mostly probably is), it is almost certainly critical in late life mortality.
- Interventions targeting repair might be most fruitful to delay ageing.

Evidence for a primary or major role of oxidative stress and damage in normal ageing is not compelling in *C. elegans*, whereas in *Drosophila* empirical support is stronger in volume, variety and consistency. This does raise questions though; what causes oxidative stress in normal living? Does environmental or dietary stress increase oxidative damage? How realistic and informative are our experiments in controlled environments?

Finally, if oxidative damage is a universal cause of a universal phenomenon, why is the evidence for its role not strong and unambiguous? The answer may lie in the multiplicity of steps and influences from ROS production to damage to ageing phenotype, resulting in inconsistent results from simple input (e.g. increased SOD) – output (lifespan) experiments. More clarity may be achieved by focusing on reducing and importantly, measuring, oxidative damage.

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