Chemical changes in aging *Drosophila melanogaster*

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Abstract The “Green Theory” of aging proposes that organismal lifespan is limited by the failure to repair molecular damage generated by a broad range of metabolic processes. Two specific predictions arise from this: (1) that these processes will produce a wide variety of stable but dysfunctional compounds that increase in concentration with age, and (2) that organisms maintained under conditions that extend lifespan will display a reduced rate of accumulation of such “molecular rubbish”. To test these predictions, novel analytical techniques were developed to investigate the accumulation of damaged compounds in *Drosophila melanogaster*. Simple preparative techniques were developed to produce digests of whole *D.*

melanogaster for use in three-dimensional (3D) fluorimetry and ^1H NMR spectrometry. Cohorts of *Drosophila* maintained under normal conditions showed an age-related increase in signals consistent with damage whereas those maintained under conditions of low temperature and dietary restriction did not. ^1H NMR revealed distinct age-associated spectral changes that will facilitate the identification of novel compounds that both increase and decrease during aging in this species. These findings are consistent with the predictions of the “Green Theory”.

Keywords *Drosophila melanogaster* · Aging · Green theory · Oxidative damage · Glycation · Spectroscopy

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Introduction

Drosophila melanogaster is a powerful model organism in which to study the aging process. As an experimental system it provides a unique combination of assets including a short lifespan, low maintenance costs, a sequenced genome and the opportunity to conduct mass screens. Both dietary restriction (DR) and mutations in insulin-IGF signalling (IIS) extend lifespan in *Drosophila* and many other species (Partridge et al. 2005; Russell and Kahn 2007). Thus, it is likely that insights into aging mechanisms that are directly relevant to higher organisms will emerge from work conducted with this model (Grandison et al. 2009; Greer and Brunet 2009).

Insulin-IGF signalling appears to be an evolutionarily conserved pathway influencing healthy lifespan (Broughton and Partridge 2009; van Heemst et al. 2005; Selman et al. 2008). Thus, understanding the mechanism by which it operates is of special interest when attempting to formulate a hypothesis with broad explanatory power for organismal aging. Analysis of the patterns of gene expression seen in the extended lifespan phenotypes of IIS mutants in multiple species has shown that successful aging is associated with significant upregulation of categories of genes linked to Phase 1 and Phase 2 xenobiotic and endobiotic metabolism (McElwee et al. 2007). This is a much broader ranging response than the simple enhancement of antioxidant defences that might have been predicted by the oxidative damage hypothesis of aging (Gems and McElwee 2005; Doonan et al. 2008). Therefore Gems and McElwee proposed that longevity assurance mechanisms involve both the removal of diverse molecular species that cause damage and the repair of damaged proteins.

This theory (dubbed the “Green Theory” of aging) proposes that aging essentially represents a ‘failure of recycling’ and could be delayed by avoiding the build up of damaged molecules and macromolecules. Under normal conditions, such “molecular rubbish” tends to accumulate in tissue because it is thermodynamically stable and energetically costly to repair. Over time this leads to the deterioration in normal tissue functions that we perceive as aging. The Green Theory predicts that states such as DR delay aging because they upregulate xenobiotic metabolism and promote the recycling or repair of damaged molecules (since organismal resources are scarce). A simplified schematic of the Green Theory is shown in Fig. 1.

Although not explicitly considered by Gems and McElwee, the non-enzymic glycation of proteins is one potential example of the type of damaging process required by the Green Theory. Non-enzymic glycation is initiated by unwanted side reactions between reducing sugars and proteins (the Maillard reaction). A cascade of complex reactions ensues, resulting in the production of both reactive damaging agents (e.g. dicarbonyl compounds) and a wide variety of chemically stable adducts and crosslinks, known collectively as advanced glycation endproducts or AGEs (Gasser and Forbes 2008). Both dicarbonyl species and AGEs themselves are substrates for cellular Phase 2 detoxification systems (Buetler et al. 2008; Hofmann et al.

Fig. 1 Simplified schematic of the “Green Theory” of aging. Aging results from a progressive failure to detoxify a broad spectrum of damaging species generated by both xenobiotic and endobiotic metabolism (A, B, C, D etc). Reduction or elimination of any one type of damaging species (e.g. oxidative damage) may have only a marginal effect on lifespan. Insulin-IGF signalling (IIS) mutants or dietary restriction (DR) increase survival by upregulating genes involved in Phase 1 and Phase 2 metabolism. This may allow “recycling” of a broad spectrum of damaged molecules that would not have been repaired under normal conditions

2001; Wenzel et al. 2002). However, AGE-adducted proteins are highly degradation resistant under normal physiological conditions due to the thermodynamic stability of the crosslinks and adducted residues. If left unrepaired, AGEs have many potentially deleterious effects, including non-competitive inhibition of enzymes and conformational deformation of proteins. In addition, it has recently been shown that a diet high in AGEs can significantly blunt the beneficial effects of DR (Cai et al. 2008).

This type of damaged molecule can be readily detected using the spectroscopic and spectrometric techniques commonly used in analytical chemistry. These have produced extensive data on the types and concentrations of various crosslinks in both healthy and diseased mammalian tissues (Sell et al. 2005; Monnier and Sell 2006). Unfortunately, they remain virtually unapplied to the study of aging in the simple model organisms. Very few studies have examined AGEs in aging *Caenorhabditis elegans* (Gerstbrein et al. 2005; Rabbani and Thornalley 2008) and data are even more sparse in *Drosophila melanogaster*. Out of almost 2,000 papers on aging in flies there is currently only one report (Oudes et al. 1998) of the use of chemical analysis to study AGEs (and even this described only single wavelength fluorimetry at just two time points, young and old). Although the analytical methods used for the detection and quantification of AGEs in mammalian tissue might simply

be adapted for use in lower organisms, Green Theory predicts that *multiple* types of stable damage will build up during aging. Thus, undue focus on any one class of compound (such as AGEs) might be inappropriate and a more sensible strategy would be the development of methods capable of surveying the broadest possible range of thermodynamically stable compounds. An advantage of this approach is that it may also lead to the identification of entirely novel age-associated chemical changes. This is particularly important since the oxidative damage theory of aging is becoming increasingly untenable in its current form (Doonan et al. 2008).

Detection of such chemical changes requires digestion of the whole organism followed by either the physical or analytical isolation of any remaining stable species. However, every sample preparation step runs the risk of loss or differential retention of the compounds of interest, or of introducing additional damage. Whilst some of these problems can be dealt with by spiking with standard samples of known compounds, many of these are not commercially available and it is not possible to quantify or control for loss of compounds that are yet to be identified. Accordingly, we have developed methods for the analysis of chemical damage that are relatively unsophisticated but that can potentially be used in non-specialist laboratories, and have a specific emphasis on reducing the complexity of sample preparation steps to maximise the recovery of stable compounds. We have applied these to *Drosophila melanogaster* aged under a range of different conditions, including DR.

Methods

Drosophila melanogaster culture, maintenance and sampling

Wild-type *D. melanogaster* (strain Dahomey) were cultured as previously described (Clancy and Kennington 2001; Bass et al. 2007). Briefly, larvae were reared at standard density in 200 cm³ glass bottles containing 70 cm³ standard sugar/yeast (SY) food. Flies emerged over 24 h and were tipped into fresh bottles and allowed 48 h to mate. Females were then separated from males under light CO₂ anaesthesia and allocated randomly to different treatments at a

density of ten females per vial. Flies were transferred to fresh vials and deaths scored three times per week. On the days of sampling, flies were transferred to 2 cm³ Eppendorf tubes without anaesthesia and the tube plunged into liquid nitrogen. Frozen samples were subsequently stored at –80°C.

Cohorts of female flies were selected for investigation, since DR has a much more substantial effect on lifespan in females than in males (Magwere et al. 2004). An initial, control cohort (25/2SY) was cultured under normal conditions (25°C and 2SY food), to generate samples to optimise analytical methods and survey the chemical changes occurring during normal aging in *D. melanogaster*. This was followed by a multiple comparison study that examined the effect of temperature and DR on the parameters identified during the initial study. This consisted of three cohorts: 25/2SY (25°C with 2SY food—control group cultured under standard conditions), 25/1SY (25°C with 1SY food—normal temperature and DR) and 18/1SY (18°C with 1SY food—low temperature and DR). Each cohort was sampled (five flies/sample) weekly to two-weekly throughout their lifespan.

Sample preparation

Each sample of five flies was defrosted, weighed and macerated in 1 cm³ 6 M HCl. Each sample was then heated to 120°C in a sealed Reacti-vial™, under N₂, for 18 h using a Reacti-Therm™ heating block. The pH was then adjusted to 8 (with 6 M NaOH), and the solution evaporated to dryness under a stream of nitrogen. Then, 600 µl D₂O (containing 0.1% TMS salt internal standard) was added to the residue, and the resulting solutions were filtered (0.5 µm syringe-mounted filter disc) and stored at –20°C until required for analysis. Preliminary spectroscopic experiments were undertaken to confirm that digests were stable and could be stored frozen for lengthy periods without affecting the resulting spectra.

Spectroscopy

A sample of each digest solution (50–200 µl) was made up to 3 ml with 0.05 M pH 8 phosphate buffer (6.80 g KH₂PO₄ dissolved in reverse osmosis water, brought to pH 8.0 with 6 M NaOH and made up to a final volume of 1 l). The resulting digest solutions

were subjected to UV absorbance spectroscopy in across the range 200–800 nm. Significant absorbances were only observed in the 280–500 nm range and this was therefore selected for the three-dimensional (3D) fluorimetry experiments. Emission spectra were recorded between 400 and 600 nm for excitation wavelengths between 280 and 500 nm (at 10 nm intervals). Linearity of response was established by subjecting the solutions to doubling dilutions and re-recording spectra. Signals due to Rayleigh scattering were excluded from the data.

Nuclear magnetic resonance spectrometry

Digest samples (in D₂O) were subjected to ¹H NMR spectrometry using a Brüker Avance 360 MHz NMR Spectrometer. In this standard characterisation technique, each proton in the mixture gives rise to a signal in the resultant spectrum. The area of each signal is proportional to the concentration of protons, and the position and complexity of each signal provides information about the local environment of any given proton. Taken as a whole, the spectrum gives information about the types and relative concentrations of the molecules present.

Results

Method development

Individual *Drosophila* are much smaller than routine mammalian tissue samples (typically 1 mg per fly), and much of the organism is composed of analytically irrelevant material such as chitin. Since it is uncertain which tissues will be most susceptible to accumulation of damaged components, and there is a premium on simplicity of sample preparation in large cohort studies, dissection was inappropriate. Oudes et al. (1998) had previously utilised trypsin digestion of macerated whole flies, and therefore this represented the initial method of choice for our work. Unfortunately, it was observed that addition of trypsin generated an overwhelming fluorescent signal (data not shown) at wavelengths within the region of interest (and adjacent to the single wavelength pair studied by Oudes et al. 1998). In order to obtain satisfactory NMR and fluorescent spectra, removal of this exogenous protein would have been required.

Such additional sample preparation steps would have increased the risk of concomitant loss of compounds of interest. The trypsin itself also represented a potential contaminant, since it could itself contain damaged molecules, and developing additional controls for such contamination was likely to be unduly complex, given that the signals observed were already close to detection limits. Accordingly, an alternative strategy was adopted.

Acid digestion has been used previously for sample preparation in AGE analysis in mammalian tissue. Although this process can lead to the loss of less stable AGEs (and other compounds), our principal interest was in stable adducts. Preparative work using acid digestion resulted in much more acceptable fluorescence data than did trypsin digestion. The improved signal-to-noise ratio from samples prepared using HCl digestion justified the selection of this method. An added advantage of using acid digests is that they are suitable for spectrophotometry and NMR spectrometry without further purification. This method therefore met our criteria for the isolation of stable compounds, with minimal preparation steps, and it was used throughout.

Aging in *Drosophila* populations

Survival curves for the cohorts used in our analyses are shown in Fig. 2. Consistent with previous studies, both temperature and DR produced marked effects on median and maximum lifespans [median (*m*) and maximum (*mx*) lifespans: 25/1SY, *m*=59.5 days, *mx*=76 days; 25/2SY, *m*=48 days, *mx*=72 days; 18/1SY, *m*=126 days. All were significantly different from each other with a max *P*-value for any pairwise comparison = 1.68×10^{-21} ; log-rank test]. This provided between *n*=6 to *n*=13 sampling points within each cohort. Fly weights were found not to differ significantly between cohorts, or with age.

Fluorimetry

Examples of 3D fluorimetric data are shown in Fig. 3a–f. Regardless of *Drosophila* age or the environmental conditions under which they are maintained, an overall maximum fluorescent intensity is observed at approximately $\lambda_{\text{ex}}=360/\lambda_{\text{em}}=440$ nm. This value is close to both that previously reported for glycated mammalian tissue (Monnier and Cerami 1981) and to the $\lambda_{\text{ex}}=365/\lambda_{\text{em}}=440$ nm employed by

Fig. 2 Survival of cohorts of *Drosophila melanogaster* comparing the effects of temperature and DR. A single cohort of *Drosophila* was reared under standard conditions and young adult females allocated to the following cohorts: 25/2SY (25°C with 2SY food—control group cultured under standard con-

ditions), 25/1SY (25°C with 1SY food—normal temperature and DR) and 18/1SY (18°C with 1SY food—low temperature and DR). Both DR and lowered temperature produced significant increases in lifespan

Fig. 3a–f Typical 3D fluorimetric emission spectra of acid digested samples of *D. melanogaster*. Intensity (fluorescence units fly⁻¹ ml⁻¹) is plotted against emission wavelength (nm). Each line represents a separate emission spectrum resulting

from excitation at the wavelength (nm) shown in the right hand key. **a** 25/2SY at 7 days, **b** 25/1SY at 7 days, **c** 18/1SY at 7 days, **d** 25/2SY at 56 days, **e** 25/1SY at 63 days, **f** 18/1SY at 147 days

Oudes et al. (1998). It is consistent with a mixture of fluorescent compounds with a substantial input from known AGEs. To allow quantitative comparison of the fluorescent spectra, data have been presented as graphs of the intensity at the spectral maximum ($\lambda_{\text{ex}}=360/\lambda_{\text{em}}=440$ nm) plotted against *Drosophila* age (Fig. 4a–c).

As shown in Fig. 4a, samples from 25/2SY *Drosophila* show a statistically significant linear increase in fluorescent intensities with age (slope= 0.44 ± 0.158 fluorescence units $\text{fly}^{-1} \text{ml}^{-1} \text{day}^{-1}$, $P=0.0131$). In contrast, samples from 18/1SY flies displayed no increase in fluorescent intensity with age (slope = -0.02 ± 0.033 fluorescence units $\text{fly}^{-1} \text{ml}^{-1} \text{day}^{-1}$, $P=0.5775$) and very little between-sample variation (Fig. 4c). Samples from 25/1SY flies show a great deal of inter-sample variability, which obscures any age-related trend in the data (slope= 0.17 ± 0.274 fluorescence units $\text{fly}^{-1} \text{ml}^{-1} \text{day}^{-1}$, $P=0.5436$, Fig. 4b).

NMR spectrometry

Although, a priori, the ^1H NMR spectra of acid-digested whole organisms might have been expected to be so complex as to defy analysis, in fact those obtained from *Drosophila* were relatively simple (a typical spectrum is shown in Fig. 5). Clearly distinguishable peaks are observed and the overall spectrum is consistent with that of a moderately complex mixture of compounds. Although signals from individual protons tend to overlap, they can be distinguished from one another, and identification of the compounds giving rise to them will thus eventually be feasible. This will require that these spectra be complemented by data generated using techniques such as mass spectrometry, but such a detailed study is far from trivial.

However, our initial qualitative analysis of the individual changes in signal size indicates that such an evaluation will be informative. Changes in the size, position and complexity of NMR signals with age indicate that both loss and gain of compounds is occurring. In addition, in order to begin to establish which signals are associated with one another (i.e. from the same molecule), an initial cluster analysis of the changes in selected regions of the spectra has been carried out.

Four regions containing peaks that were consistently identifiable within all spectra were selected (see Fig. 5)

Fig. 4a–c Fluorescence intensity ($\lambda_{\text{ex}}=360/\lambda_{\text{em}}=440$ nm) vs age for female *Drosophila* cultured under different conditions. **a** 25/2SY, **b** 25/1SY, **c** 18/1SY. Each data point was generated from a pooled digest of $n=5$ individual flies. The lines of best fit generated by unweighted linear regression are shown. Slopes and statistical significance are given in the text

Fig. 5 Typical 360 MHz ^1H NMR spectrum of an acid digest sample, showing all significant signals. Regions selected for further analysis are labelled 1–4 and discussed in the text

and the area under these peaks in these regions quantified relative to the TMS salt peak at 0.0 ppm. These areas were then plotted against age of fly at sampling and the line of best fit was found by unweighted linear regression. This generated slope and intercept values (with associated errors) for each region and culture condition. In order to identify peak regions whose size or rate of change are correlated, the intercept (representing the initial concentration of protons giving the signal) was plotted against the slope (proxy for rate of change of this concentration) for each of the selected regions and culture conditions (see Fig. 6). This pinpoints both associations between regions (those that co-vary) and differences between spectral changes associated with altered culture conditions. At this level of detail there are strong indicators that both the size and rate of change of peaks in regions 1 and 3 co-associate, as do regions 2 and 4 (in particular see Fig. 6a, showing two distinct pair of data points). This suggests that the main underlying signals in these two sets of data are from the same molecule, or are from molecules that are equivalently affected by age.

The ^1H NMR signals generated by samples from 25/2SY flies show a distinct reduction in size in all four of the selected regions with age of cohort at

sampling. In contrast, signals from 18/1SY fly samples display a relatively high initial intensity (intercept) similar to that of young 25/2SY flies, and no significant change in this intensity with age. As was also observed using fluorimetry, 25/1SY flies display a great deal more inter-sample variability than the 18/1SY cohort.

Discussion and conclusions

We have conducted the first multi-time point analysis of chemical changes in *Drosophila* aged under a range of temperatures and nutrient levels. Our 3D fluorimetric data confirm and extend the initial observation by Oudes et al. (1998) that stable fluorescent chemical species accumulate with age in fully fed flies. In contrast, flies maintained under conditions of DR and low temperature show no significant accumulation of these species. We attribute this to a combination of increased rates of protein repair and decreased rates of endobiotic damage (as a result of lowered metabolic activity). Interestingly, we observe no increase in fluorescence at the end of lifespan in this cohort. Similarly, Oudes

Fig. 6 Cluster analysis of changes in the area under ^1H NMR peak clusters 1–4 for the three culture conditions (**a** 25/2SY, **b** 25/1SY, **c** 18/1SY). Each data point was generated by examining the integral for one of four selected clusters and

generating the line of best fit by unweighted regression. The resultant slope and y-intercept, together with their associated errors were then plotted on the x and y axis respectively

et al. (1998) demonstrated that treatment of fully fed *Drosophila* with aminoguanidine significantly reduced endogenous fluorescence (probably AGEs) but failed to increase mean lifespan. There are two possible explanations for these data, which are not mutually exclusive.

The first of these is that we have reduced or eliminated a major cause of pathology in *Drosophila* that are maintained under standard conditions, only to replace it with a different primary cause of death for those maintained under conditions of low temperature and DR. The efficiency of the immune system in *Drosophila* is known to decline during ageing (Ramsden et al. 2008) but it has recently been demonstrated that nutrient restricted aged rodents are more likely to die from infection than ad libitum fed age-matched controls (Ritz and Gardner 2006). Thus, infection may represent a plausible primary cause of death in our long-lived cohort.

The second possibility is that, as the “Green Theory” proposes, lifespan is set by the efficiency with which *multiple* types of damaged macromolecules are recycled. If this is correct, then reducing the rate of formation of any single type of damage would be unlikely to have more than a marginal effect on survival. This would explain both the failure of Oudes et al. (1998) to extend lifespan using aminoguanine and the observation by Mockett et al. (2003) that ectopic elevation of catalase activity fails to increase lifespan in *Drosophila* but does significantly increase stress resistance. Thus, it is possible that death occurs in the long-lived cohort as a result of damaging molecular changes that cannot be detected by fluorescence. To begin to identify novel chemical species within *Drosophila*, such that a more complete spectrum of the chemical changes occurring during aging can be produced, we undertook ^1H -NMR analysis of our aging flies.

An initial qualitative evaluation of our NMR spectra indicates that individual compounds are both gained and lost during the process of ageing in *Drosophila* (an observation consistent with traditional histology in this species, Miquel 1971). Four clusters of peaks are consistently identifiable in all spectra, and this has allowed us to quantify the changes in the area under the peak clusters, and thus determine the relative concentrations of molecules giving rise to them. Our quantitative data are consistent with the presence of a molecule (or set of molecules) within

young cohorts of flies which is gradually lost at different rates as they age. However, identification of the chemical species giving rise to these spectral changes will require additional instrumental analysis. We are currently undertaking this.

25/1SY flies give rise to samples which show elevated scatter compared to the other cohorts studied. This scatter is observed using both ^1H NMR spectrometry and fluorimetry. The most likely reason for this is that elevated rates of endobiotic damage (as a result of higher metabolic rates) and of detoxification (as a consequence of being in a DR state) are acting in combination. Since both the relative and absolute variance of rates of enzyme-catalysed reactions increase with rate (Askelöv et al. 1976) the inter-sample variation rises dramatically if both processes are accelerated. In theory, increasing cohort size could compensate for the increased scatter, allowing any trends to be identified. Practically, however, the cohorts we have used are already large and increasing these would render studies unwieldy.

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References

- Askelöv P, Korsveldt M, Mannervik B (1976) Error structure of enzyme kinetic experiments, implications for weighting in regression analysis of experimental data. *Eur J Biochem* 69:61–67
- Bass TM, Grandison RC, Wong R, Martinez P, Partridge L, Piper MD (2007) Optimization of dietary restriction protocols in *Drosophila*. *J Gerontol A Biol Sci Med Sci* 62(10):1071–1081
- Broughton S, Partridge L (2009) Insulin/IGF-like signalling, the central nervous system and aging. *Biochem J* 418(1):1–12
- Buetler TM, Latado H, Baumeier A, Delatour T (2008) Dicarbonyls stimulate cellular protection systems in primary rat hepatocytes and show anti-inflammatory properties. *Ann N Y Acad Sci* 1126:113–117
- Cai W, He JC, Zhu L, Chen X, Zheng F, Striker GE, Vlassara H (2008) Oral glycotoxins determine the effects of calorie restriction on oxidant stress, age-related diseases, and lifespan. *Am J Pathol* 173:327–336
- Clancy DJ, Kennington WJ (2001) A simple method to achieve consistent larval density in bottle cultures. *Dros Inf Serv* 84:168–169

- Gasser A, Forbes JM (2008) Advanced glycation: Implications in tissue damage and disease. *Protein Pept Lett* 15:385–391
- Doonan R, McElwee JJ, Matthijssens F, Walker GA, Houthoofd K, Back P, Matscheski A, Vanfleteren JR, Gems D (2008) Against the oxidative damage theory of aging: superoxide dismutases protect against oxidative stress but have little or no effect on life span in *Caenorhabditis elegans*. *Genes Dev* 22(23):3236–3241
- Gems D, McElwee JJ (2005) Broad spectrum detoxification: the major longevity assurance process regulated by insulin/IGF-1 signaling? *Mech Age Dev* 126:381–387
- Gerstbrein B, Stamatatos G, Kollias N, Driscoll M (2005) In vivo spectrofluorimetry reveals endogenous biomarkers that report healthspan and dietary restriction in *Caenorhabditis elegans*. *Aging Cell* 4:127–137
- Grandison RC, Wong R, Bass TM, Partridge L, Piper MD (2009) Effect of a standardised dietary restriction protocol on multiple laboratory strains of *Drosophila melanogaster*. *PLoS ONE* 4(1):e4067
- Greer EL, Brunet A (2009) Different dietary restriction regimens extend lifespan by both independent and overlapping genetic pathways in *C. elegans*. *Aging Cell* 8:113–127. doi:10.1111/j.1474-9726.2009.00459.x
- Hofmann T, Ames J, Krome K, Faist V (2001) Determination of the molecular weight distribution of non-enzymatic browning products formed by roasting of glucose and glycine and studies on their effects on NADPH-cytochrome *c*-reductase and glutathione-S-transferase in Caco-2 cells. *Nahrung* 45(3):189–194
- Magwire T, Chapman T, Partridge L (2004) Sex differences in the effect of dietary restriction on life span and mortality rates in female and male *Drosophila melanogaster*. *J Gerontol A Biol Sci Med Sci* 59:B3–B9
- McElwee JJ, Schuster E, Blanc E, Piper MD, Thomas JH, Patel DS, Selman C, Withers DJ, Thornton JM, Partridge L, Gems D (2007) Evolutionary conservation of regulated longevity assurance mechanisms. *Genome Biol* 8:R132
- Miquel J (1971) Ageing of male *Drosophila melanogaster*: Histological, histochemical and ultrastructural observations. *Adv Gerontol Res* 3:39–71
- Mockett RJ, Bayne AC, Kwong LK, Orr WC, Sohal RS (2003) Ectopic expression of catalase in *Drosophila* mitochondria increases stress resistance but not longevity. *Free Radic Biol Med* 34(2):207–217
- Monnier VM, Cerami A (1981) Nonenzymatic browning in vivo: possible process for aging of long-lived proteins. *Science* 211(4481):491–493
- Monnier VM, Sell DR (2006) Prevention and repair of protein damage by the Maillard reaction in vivo. *Rejuvenation Research* 9:264–273
- Oudes AJ, Herr CM, Olsen Y, Fleming JE (1998) Age-dependent accumulation of advanced glycation end-products in adult *Drosophila melanogaster*. *Mech Aging Dev* 100:221–229
- Partridge L, Pletcher SD, Mair W (2005) Dietary restriction, mortality trajectories, risk and damage. *Mech Ageing Dev* 126(1):35–41
- Rabbani N, Thornalley PJ (2008) Dicarboxyls linked to damage in the powerhouse: glycation of mitochondrial proteins and oxidative stress. *Biochem Soc Trans* 36:1045–1050
- Ramsden S, Cheung YY, Seroude L (2008) Functional analysis of the *Drosophila* immune response during aging. *Aging Cell* 7(2):225–236
- Ritz BW, Gardner EM (2006) Malnutrition and energy restriction differentially affect viral immunity. *J Nutr* 136:1141–1144
- Russell SJ, Kahn CR (2007) Endocrine regulation of ageing. *Nature Rev Mol Cell Biol* 8:681–691
- Sell DR, Biemel KM, Reihl O, Lederer MO, Strauch CM, Monnier VM (2005) Glucosepane is a major protein cross-link of the senescent human extracellular matrix. Relationship with diabetes. *J Biol Chem* 280:12310–12315
- Selman C, Lingard S, Choudhury AI, Batterham RL, Claret M, Clements M, Ramadani F, Okkenhaug K, Schuster E, Blanc E, Piper MD, Al-Qassab H, Speakman JR, Carmignac D, Robinson IC, Thornton JM, Gems D, Partridge L, Withers DJ (2008) Evidence for lifespan extension and delayed age-related biomarkers in insulin receptor substrate 1 null mice. *FASEB J* 22(3):807–818
- van Heemst D, Beekman M, Mooijaart SP, Heijmans BT, Brandt BW, Zwaan BJ, Slagboom PE, Westendorp RG (2005) Reduced insulin/IGF-1 signalling and human longevity. *Aging Cell* 4(2):79–85
- Wenzel E, Tasto S, Erbersdobler HF, Faist V (2002) Effect of heat-treated proteins on selected parameters of the biotransformation system in the rat. *Ann Nutr Metab* 46(1):9–16