Chemical analysis of aging: testing predictions from Green Theory

Elizabeth L. Ostler,[1] Aamira Iqbal,[1] Richard G.A. Faragher,[1] Declan Naughton[2] Matthew Piper[3] and Linda Partridge.[3]

[1] School of Pharmacy and Biomolecular Sciences, University of Brighton, Lewes Road, Brighton. UK. email: e.ostler@bton.ac.uk

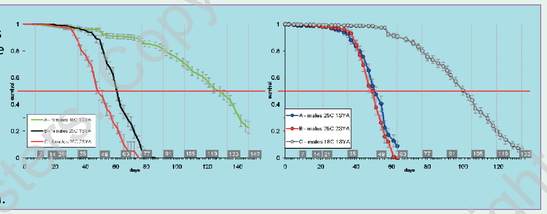
[2] School of Life Sciences, Kingston University, Penrhyn Road, Kingston-upon-Thames. UK. [3] Dept of Biology, University College, Gower St., London. UK.

Drosophila culture

Fly stocks, maintenance and handling procedures - All experiments were performed with the wild-type, outbred, laboratory strain Dahomey unless otherwise stated. The population is maintained in large population cages with overlapping generations on a 12h:12h light:dark cycle at 25°C or 18°C and 65% humidity. Standard lab food was prepared on a gas hob using 100g lyophilized yeast, 50 g sugar and 15g agar per litre. After boiling all components together, preservatives were added and food dispensed into glass vials.

Fly rearing, maintenance and sampling - Larvae were reared at standard density in 200ml glass bottles containing 70ml of 1.0 SY or 2.0SY food.[3] Flies emerged over 24h and were tipped into fresh bottles and allowed 48h to mate. Females were then separated from males under light CO_2 anaesthesia and randomly allocated to different treatments at a density of 10 flies per vial. Flies were transferred to fresh vials and deaths scored at least every two days. On the days of sampling, flies were transferred to eppendorf tubes without anaesthesia and the tube plunged into liquid nitrogen. Frozen samples were subsequently stored at -80°C.

Figure 1: Survival of cohorts of female Drosophila melanogaster comparing the effects of temperature and dietary restriction. A single cohort of Drosophila were reared under standard conditions and young adult females allocated to the treatments indicated (1SY are dietary restricted, 2SY are fully fed, normal temperature = 25˚C, low temperature = 18˚C). Both dietary restriction and lowered temperature produced significant increases in lifespan in females, but only low temperature conditions significantly increased male lifespan.

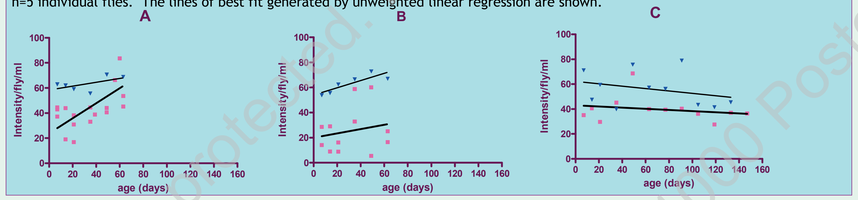


Sample Preparation

6M HCl digests of whole *Drosophila* were found to give more reproducible results and less interference problems than enzyme-mediated hydrolysates. A typical protocol is as follows. Five flies were defrosted, weighed and then macerated with 0.5cm^3 6M HCl. The homogenised fly samples were then placed in Reacti-Vials™, degassed with nitrogen, sealed and heated to 100°C for 18h. The acid-hydrolysed samples were then neutralized with 6M NaOH and warmed to 40°C for evaporation in a stream of nitrogen. After evaporation the residue was redissolved in a minimum volume of D_2O and stored at -20°C until required for further analysis. Samples for Mass Spectrometry were further purified using C18 microSPE cartridges to remove NaCl.

Fluorimetry

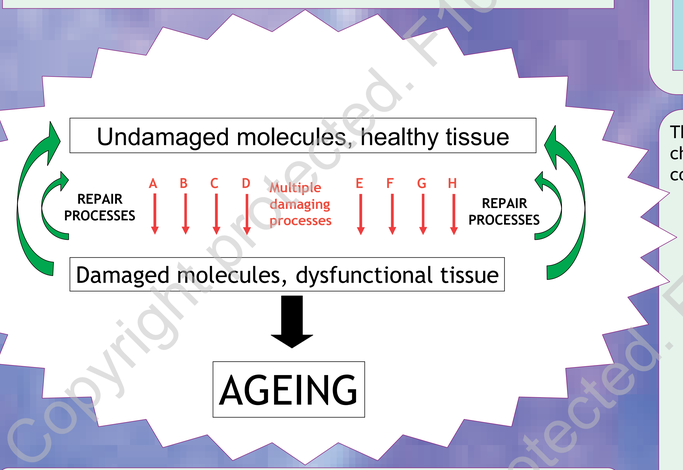
Figure 2: Fluorescence intensity (λ_{ex}=360/λ_{em}=440nm) versus age for male (blue triangles) and female (pink squares) Drosophila cultured under different conditions: A=25°C, 2SY, B=25°C, 1SY, C=18°C, 1SY. Each data point was generated from a pooled digest of n=5 individual flies. The lines of best fit generated by unweighted linear regression are shown.



Samples from fully fed female Drosophila cultured at 25°C show a statistically significant linear increase in fluorescence intensities with age (Figure 2A Slope = 0.44 ± 0.158 fluorescence units.fly^{-1}.ml^{-1}.day^{-1}, p = 0.0131). In contrast, samples from dietary restricted female flies cultured at 18°C displayed no increase in fluorescent intensity with age (Slope = -0.02 ± 0.033 fluorescence units.fly^{-1}.ml^{-1}.day^{-1}, p = 0.5775) and very little between-sample variation (Figure 2C). Samples from flies cultured at 25°C under conditions of dietary restriction show a great deal of inter-sample variability, which obscures any age-related trend in the data (Slope = 0.17 ± 0.274 fluorescence units.fly^{-1}.ml^{-1}.day^{-1}, p = 0.5436, Figure 2B). Digests of male flies exhibited significantly (p<0.001) higher fluorescence than that from females at early time points, and no significant trends.

SUMMARY

Theories of aging based on simple oxidative damage have been unsuccessful in explaining extended lifespan. Gems *et al* have proposed[1] that broad spectrum detoxification provides a more satisfactory explanation of lifespan extension by dietary restriction and IIS mutation. This "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 (See schematic below). To test this theory, we developed novel methods for the chemical analysis of whole *Drosophila melanogaster*. These methods were designed to survey the broadest possible range of stably damaged compounds and to be usable in laboratories that lack routine access to sophisticated analytical purification techniques. When applied to *Drosophila* aged under a range of dietary and environmental conditions the data proved to be incompatible with aging due to simple oxidative damage but consistent with Green theory. This poster reports an extension of this published work.[2]



SIMPLIFIED SCHEMATIC OF GREEN THEORY

3D Fluorimetry

50-300µl of hydrolysate was made up to 3ml with 0.05M pH8 phosphate buffer. Doubling dilutions were used to check linearity of response. Two dimensional scans of λ_{ex} 300-500nm and λ_{em} 400-600nm were recorded. Signals due to Rayleigh scattering were excluded from the presented data for clarity.

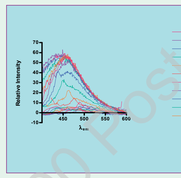


Figure 3: Typical 3-D Fluorimetric emission spectrum of an acid digested sample of Drosophila melanogaster. Intensity (fluorescence units.fly^{-1}.ml^{-1}) is plotted against emission wavelength (nm). Each line represents a separate emission spectrum resulting from excitation at the wavelength (nm) shown in the right hand key

Nuclear Magnetic Resonance Spectrometry

Proton NMR spectra were recorded under standardised conditions. TMS salt was used both as a qualitative and quantitative internal standard. Spectra were divided into 11 regions as shown below, and the area under each cluster divided by that of TMS salt to give a relative value for the concentration of protons present in the sample with signals in that region.

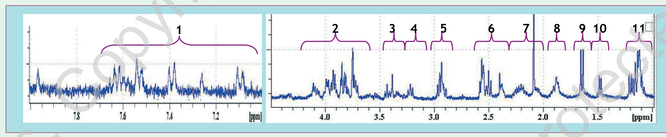
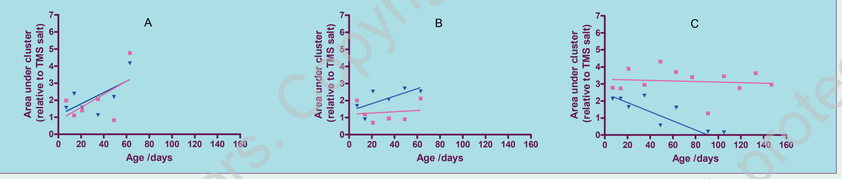


Figure 4: Typical 360MHz proton NMR signals from an acid digest sample, showing all significant signals, marked to show the 11 regions whose integrals were used in the subsequent trend analysis

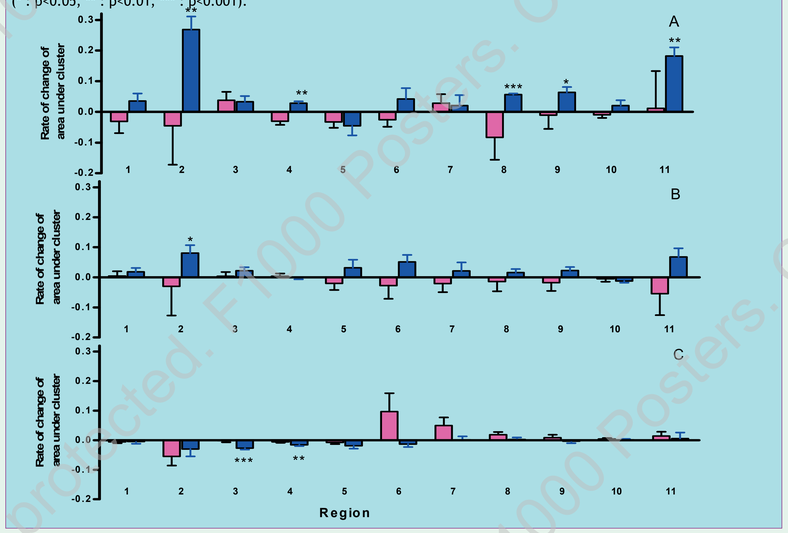
The area under the clusters was then plotted against age of cohort to identify any age-related trends in signal size. An example of such plots for the peaks in cluster 4 is shown in figure 5.

Figure 5: Area under ^{1}H NMR cluster 4 versus age for male (blue triangles) and female (pink squares) *Drosophila* cultured under different conditions: A=25°C, 2SY, B=25°C, 1SY, C=18°C, 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.



The age-related trends for each cohort, for all of the 11 clusters, can be compared by plotting these as a bar chart, with their associated error ($S_{y/x}$). These show the rate of change in the concentrations of proton-containing compounds present in the digests.

Figure 6: Rate of change of area under ^{1}H NMR signal clusters versus age for male (blue) and female (pink) *Drosophila* cultured under different conditions: A=25°C, 2SY, B=25°C, 1SY, C=18°C, 1SY. The error bars represent the error in the slope ($s_{y/x}$). Stars represent a slope which displays an age associated trend by differing significantly from horizontal (* : p<0.05, ** : p<0.01, *** : p<0.001).



Peaks in regions 2, 4, 8, 9 & 11 showed significant age associated increase in male fully-fed flies at 25°C. This trend was reduced or abolished in the matched DR cohort. Culture at low temperature under DR conditions abolished any age-associated increases and induced a small but significant age-associated decrease in signal strength in regions 3 and 4. Peaks in regions 5-7 and 10 displayed no discernable trends. Female cohorts gave rise to signals which followed the same trends, but were not statistically significant.

REFERENCES:1. Gems D & McElwee JJ (2005) Mech Age Dev **126**:381-387; 2. Iqbal A *et al*. (2009) AGE **31**, 343. 3. DJ Clancy &WJ Kennington (2001) *Dros. Inf. Serv.* **84**:168-169.