

Biomarkers of aging in *Drosophila*

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Summary

Low environmental temperature and dietary restriction (DR) extend lifespan in diverse organisms. In the fruit fly *Drosophila*, switching flies between temperatures alters the rate at which mortality subsequently increases with age but does not reverse mortality rate. In contrast, DR acts acutely to lower mortality risk; flies switched between control feeding and DR show a rapid reversal of mortality rate. Dietary restriction thus does not slow accumulation of aging-related damage. Molecular species that track the effects of temperatures on mortality but are unaltered with switches in diet are therefore potential biomarkers of aging-related damage. However, molecular species that switch upon instigation or withdrawal of DR are thus potential biomarkers of mechanisms underlying risk of mortality, but not of aging-related damage. Using this approach, we assessed several commonly used biomarkers of aging-related damage. Accumulation of fluorescent advanced glycation end products (AGEs) correlated strongly with mortality rate of flies at different temperatures but was independent of diet. Hence, fluo-

rescent AGEs are biomarkers of aging-related damage in flies. In contrast, five oxidized and glycated protein adducts accumulated with age, but were reversible with both temperature and diet, and are therefore not markers either of acute risk of dying or of aging-related damage. Our approach provides a powerful method for identification of biomarkers of aging.

Key words: biomarkers of aging; demography of aging; *Drosophila*.

Introduction

Aging is characterized by accumulation of damage to molecules, cells, tissues and the systemic environment, leading to loss of function, increasing vulnerability to aging-related diseases and eventual death (Kirkwood, 2005; Vijg & Campisi, 2008). Because the aging process is slow, biomarkers of aging that can predict differences between individuals in time to death have long been sought, but have proved elusive (Warner, 2004).

Ectotherms, including the fruit fly *Drosophila melanogaster*, generally live longer at lower ambient temperatures within the physiological range (Loeb & Northrop, 1917). Mortality patterns in such experiments can be examined in detail by fitting data to a Gompertz model of mortality ($\mu_x = ae^{bx}$) where μ_x represents mortality rate at age x , a is the baseline mortality and b is the change in mortality rate with age, i.e. the slope of the mortality trajectory. The mortality rate (μ_x) of flies maintained at higher temperature (27 °C) increases more rapidly with age than does that of flies kept at a lower temperature (18 °C) (Pletcher *et al.*, 2000; Mair *et al.*, 2003), and b is therefore greater. Presumably, as for other biochemical processes, those causing aging-related damage increase in rate at higher temperatures. Consistent with this idea, switching flies between thermal regimes alters only the rate at which mortality subsequently increases with age (b) to that characteristic of the new thermal regime (Mair *et al.*, 2003). The flies are thus permanently affected by their thermal history, and they have more aging-related damage predictive of death with a longer thermal history at higher temperature.

Dietary restriction (DR) also extends the lifespan of taxonomically diverse organisms, including budding yeast *Saccharomyces cerevisiae* (Jiang *et al.*, 2000; Lin *et al.*, 2004), the nematode worm *Caenorhabditis elegans* (Klass, 1977; Houthoofd *et al.*, 2003), the fruit fly *D. melanogaster* (Chapman & Partridge, 1996), rats (McKay *et al.*, 1935), mice (Weindruch *et al.*, 1986) and primates (Colman *et al.*, 2009). Methods of restricting dietary intake necessarily differ for different organisms, but with food dilution (see Experimental Procedures), mortality rate (μ_x) in *Drosophila* is reliably reduced (Chapman & Partridge, 1996; Mair *et al.*, 2003) with no compensatory increase in feeding rate (Wong *et al.*, 2008, 2009). This widespread occurrence may implicate DR as one of the few known 'public mechanisms' that

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modulate longevity (Partridge & Gems, 2002). In striking contrast to the effects of temperature, DR acts acutely to lower death rate (μx) in *Drosophila* by decreasing baseline mortality (a) rather than by changing the slope of the mortality trajectory (b) (Mair et al., 2003). Mortality rates (μx) of flies that are switched between full feeding and DR also switch completely to those of flies kept permanently on the new feeding regime (Mair et al., 2003). This reversal of risk can be induced at any adult age. Several molecular traits and cancer incidence respond rapidly to reversals of DR status in the mouse (Hurting et al., 2003; Spindler, 2005), but the effects upon mortality have not yet been reported. Thus, DR in *Drosophila* lowers overall mortality rates (μx), but does not slow the rate of increase in mortality rate with age (b , the slope of the mortality trajectory), generally thought to be reflective of the rate of aging (Mair et al., 2003). At least in mice, DR also lowers overall mortality (μx) and baseline mortality (a) rather than lowering the rate of increase with age (b) (Partridge et al., 2005), a possible indication that, as in *Drosophila*, risk of death, rather than accumulation of aging-related damage, is lowered by DR.

The contrasting effects on mortality rate (μx) of temperature and DR provide a potentially powerful method for detecting biomarkers of aging in *Drosophila*. Markers of the irreversible damage associated with aging would be predicted to accumulate with age and to be refractory to a change in diet, and would instead track the effects of temperature on mortality rates, and would therefore not reverse upon switching flies to a different thermal environment (Partridge et al., 2005). In contrast, damaged molecular species associated with diet and the risk of dying should be fully reversible with the onset or cessation of DR, within the timescale of the mortality switch. We have used this approach to assess some candidate biomarkers of aging.

Advanced glycation end products (AGEs) are formed from the nonenzymatic reaction between reducing sugars and amine residues on proteins, lipoproteins or nucleic acids. Multiple pathways give rise to AGEs, including the Maillard reaction, Schiff base formation and Amadori rearrangements (Baynes, 2001), and they form part of a wider group of inter-related, age-related protein modifications including lipofuscin, ceroid, age pigments and age pigment-like fluorophores (Yin, 1996). Despite their complex aetiology, some AGEs exhibit a characteristic fluorescence that can be measured by relatively simple techniques. Fluorescent AGE product accumulation has been measured in various organisms, including yeast (Reverter-Branchat et al., 2004), *C. elegans* (Gerstbrein et al., 2005), *Drosophila* (Miquel et al., 1974; Oudes et al., 1998) and zebrafish (Kishi et al., 2008). Oxidative macromolecular damage has also been much studied in aging animals (Hulbert et al., 2007; Pamplona & Barja, 2007) and an increase in steady state levels with age of a variety of specific protein and carbohydrate adducts has been demonstrated in various taxa (Sohal & Dubey, 1994; Levine & Stadtman, 2001; Pamplona et al., 2002; Grzelak et al., 2006). Glutamic semialdehyde (GSA) derives from the metal-catalysed oxidation of proline and arginine, and amino adipic semialdehyde (AASA) results from lysine oxidation. These are among the main carbonyl

products of metal-catalysed oxidation of proteins and are thus specific probes of oxidation of amino acids in protein. Carbohydrates, when reacting with free radicals, generate highly reactive carbonyl compounds, such as glyoxal and methylglyoxal. These reactive carbonyl species generate stable adducts with lysine, arginine and cysteine in proteins. *N*-(Carboxyethyl)-lysine (CEL) and *N*-(carboxymethyl)-lysine (CML) are two of these (Pamplona et al., 2005). Finally, *N*-malondialdehyde-lysine (MDAL) is the product of the interaction of malondialdehyde (derived from lipid peroxidation) and lysine residues. Taken together, measurements of these markers provide an overview of the oxidative state of a range of key proteins and in the aging animal.

In this study we analysed how levels of fluorescent AGEs and five specific oxidized protein adducts (GSA, AASA, CEL, CML, and MDAL) change with age and with switches in temperature and diet in *Drosophila*. All of the markers increased with the chronological age of the flies in a standard environment, implying that all could potentially act as biomarkers of aging. As would be required for such a biomarker, fluorescent AGEs were unresponsive to diet but tracked the effects of temperature upon mortality, and they thus accumulate as part of the aging process itself. However, surprisingly and in striking contrast, all of the specific oxidized protein adducts responded to both diet and temperature with a complete switch in levels, implying that they are neither part of the aging process nor predictive of risk of death. Instead, they act as markers of the acute thermal or dietary status of the flies. They may thus act as markers of acute changes in state with age, and they clearly are not markers of the aging process itself.

Results

Mortality trajectories

The temperature switch mortality results (Fig. 1A) are representative of responses that have proved highly repeatable. Flies maintained at 18 °C ($n = 3843$) survived significantly longer than those maintained at 27 °C ($n = 5146$; $P < 0.0001$, Log-Rank test). Data were fitted to the Gompertz model ($\mu x = ae^{bx}$) where μx represents mortality rate at age x , a is the baseline mortality and b is the change in mortality rate with age, i.e. the slope of the mortality trajectory (μx is illustrated in Fig. 1A,B, while factors a and b are reported in Tables S1 and S2). The intercept of the mortality trajectory (a) with the ordinate was indistinguishable for flies chronically housed at either 27 or 18 °C, indicating that both groups start from the same, low level risk of death. When flies were switched from high to low temperature ($n = 1540$), the slope of the mortality trajectory (b) decreased and became indistinguishable from that of flies chronically maintained at 18 °C (Table S1). Thus, the mortality rates (μx) of the switched flies were permanently higher than those of the chronic 18 °C group (Mair et al., 2003). Conversely, when flies maintained at 18 °C were switched to 27 °C, the slope, b , of the subsequent mortality trajectory became similar to that of the chronic 27 °C group, and the switched flies

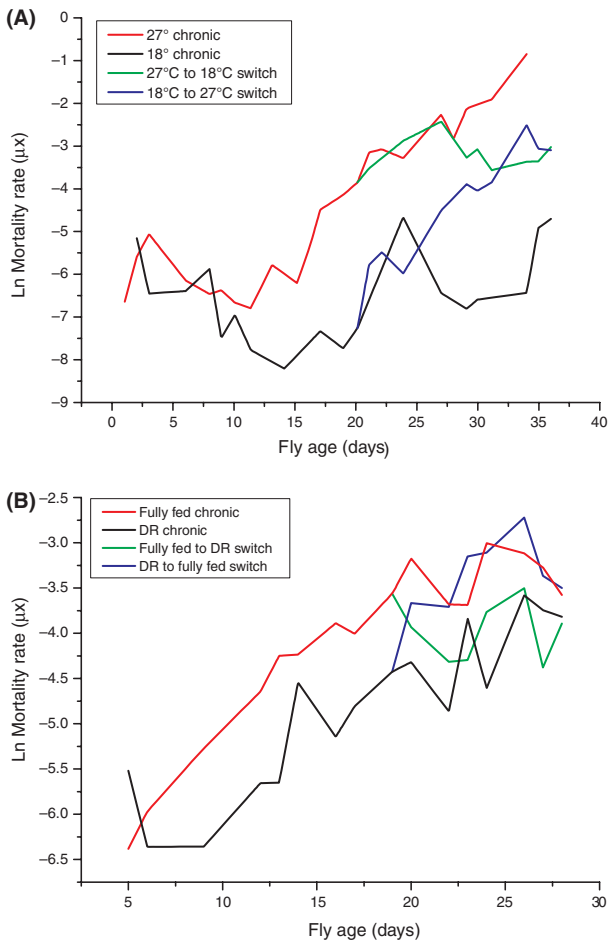


Fig. 1 Effects of temperature and dietary restriction (DR) on *Drosophila* mortality. (A) Mortality rates of flies housed at different temperatures. Age-specific mortality analysis of lifespan data from once-mated female flies was performed ($n = 5146$ at 27°C , $n = 3843$ at 18°C , $n = 1540$ switched from 27°C to 18°C and $n = 2026$ for the reciprocal switch). The initial rate of mortality (a) was statistically indistinguishable for flies chronically housed at either temperature but the rate of mortality increase with age (b , the slope of the mortality trajectory), was significantly different between the two groups (see text, Table S1). Switching flies between temperatures altered the mortality trajectory slopes but the age-specific mortality rates of the switched groups always remained distinct from those of the chronic groups. (B) Mortality rates of fully fed flies or flies undergoing DR. Male flies were maintained on full feed ($n = 1184$) or DR ($n = 1171$) or switched between the regimes ($n = 464$ switched from control to DR, $n = 515$ for the reciprocal switch). Age-specific mortality analysis of lifespan data was performed, and a Gompertz model was fitted to the linear portion of the mortality curves. The initial rate of mortality (a , the intercept with the ordinate) was significantly different in flies subjected to DR (see text, Table S2). Switched flies rapidly adopted the age-specific mortalities of flies on chronic feeding regimes.

continued to have lower mortality rates (μx) and lower extrapolated intercept (a) than the chronic 27°C group (Table S1). It can therefore be concluded that flies switched to 18°C started with the same risk of death as those at 27°C but then aged more slowly.

The mortality trajectories of flies on DR or control food (Fig. 1B) also resembled those from earlier studies (Mair et al., 2003), with a lowering of the intercept of the mortality trajectory, a , by DR and a complete reversal of mortality rates, μx , with

a switch in diet (Mair et al., 2003). Statistical analysis of a Gompertz model fitted to the trajectories (Table S2) showed that the intercept, a , with the ordinate of the mortality trajectory of flies subjected to DR ($n = 1171$) was significantly lower than that of the fully fed cohort ($n = 1184$). However, in contrast to temperature, the rates of increase in the age-specific mortalities, b , were statistically indistinguishable from one another. Flies switched from fully fed to a DR diet ($n = 464$) adopted a mortality rate, μx , that overlapped that of the chronic DR cohort (Fig. 1B). The reverse switch ($n = 515$) induced an immediate increase in mortality rate that overlapped and then surpassed that of the fully fed cohort (Fig. 1B).

Fluorescent AGEs in temperature switch flies

There was a clear difference in the AGE content of flies maintained at different temperatures (Fig. 2A). At all time points, flies maintained at 27°C had significantly higher levels of AGEs than those kept at 18°C ($P < 0.0001$, one-way ANOVA with Tukey–Kramer Multiple Comparisons *post hoc* test, $n = 3$) and the rate of AGE accumulation in flies at 27°C was significantly greater than those at 18°C ($F = 58.2305$, $\text{DFn} = 1$, $\text{DFd} = 8$, $P < 0.0001$; analysis of covariance). When flies were switched from 27 to 18°C , the AGE accumulation rate slowed to that of flies maintained at 18°C throughout the experiment (slopes 0.0033 ± 0.0017 and 0.0056 ± 0.0006 respectively). The reciprocal switch induced an immediate increase in rate of AGE accumulation to a rate that was indistinguishable from that of the chronic 27°C group (slopes 0.0145 ± 0.0013 and 0.0151 ± 0.0011 , respectively). Moreover, plotting fluorescent AGE content against mortality rates (μx) of flies maintained chronically at either temperature (Fig. 2B) revealed a single mathematical relationship between mortality rate and AGEs, because neither intercepts ($F = 0.3229$, $\text{DFn} = 1$, $\text{DFd} = 15$, $P = 0.58$) nor slopes ($F = 0.0009$, $\text{DFn} = 1$, $\text{DFd} = 14$, $P = 0.98$; analysis of covariance) were different in either chronic or switched cohorts, confirming that accumulation of these fluorescent AGEs reflects the temperature-dependent mortality rate. Switching flies between temperatures altered neither the slopes, b , nor the intercepts, a , of the regressions of either group (Fig. 2B).

Fluorescent AGEs in DR switched flies

Fluorescent AGEs increased with age in flies maintained on both diets, but there was no significant difference in fluorescent AGEs between dietary groups at any age ($P > 0.05$, Fig. 2C). There was therefore no effect of switching dietary regimes on AGE accumulation. Figure 2(D) illustrates the relationship between mortality rate, μx , and AGE signal in chronic control and DR flies where the intercepts, a , of the regression lines are significantly different ($P < 0.001$) but there was no statistical difference between the slopes of the regressions. Notably, the slopes ($F = 1.60569$, $\text{DFn} = 1$, $\text{DFd} = 5$, $P = 0.26$ for controls, r^2 for the switched flies = 0.96, analysis of covariance) and intercepts ($F = 0.621817$, $\text{DFn} = 1$, $\text{DFd} = 6$, $P = 0.46$ for controls)

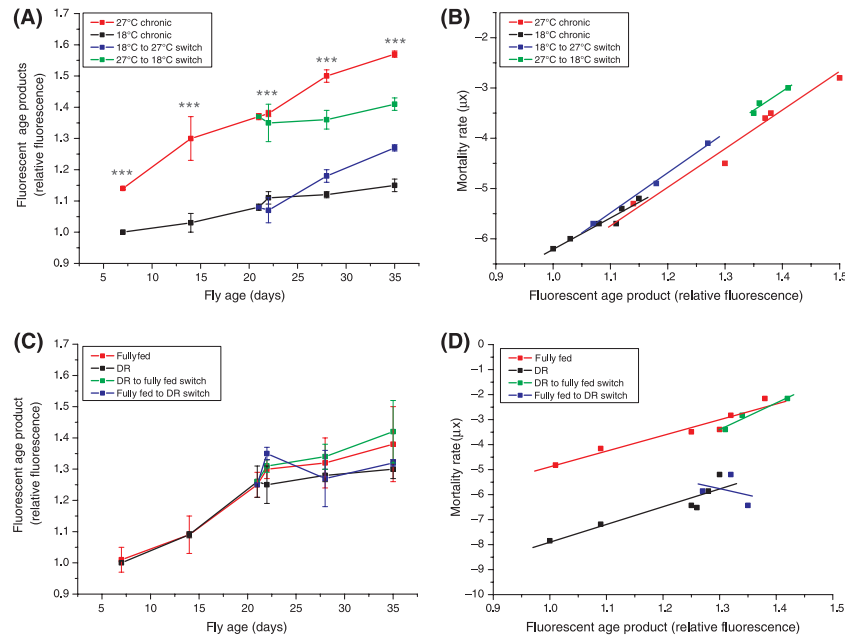


Fig. 2 Fluorescent advanced glycation end products (AGE) accumulation reflects temperature-dependent mortality but is independent of diet. (A) Fluorescent AGE products accumulated with chronological age in flies housed at different temperatures (20 flies per sample, $n = 3$ samples for each condition). At all time points, flies chronically housed at 27 °C had higher AGE levels than those at 18 °C. Switching flies to 27 °C induced an immediate slowing in AGE accumulation to a rate indistinguishable from flies chronically maintained at 18 °C but AGE accrual was irreversible in the time scale of this experiment. The reverse switch (18–27 °C) induced an immediate rise in AGE accumulation rate to that of flies kept chronically at 27 °C but total AGE content never reached that of flies chronically maintained at 27 °C. *** $P < 0.001$. (B) There was no significant difference in either slope or intercept in regression lines fitted to plots of μx (Fig. 1B) against AGE content for either chronic or switched cohorts. The data are best described by a single mathematical relationship. (C) Fluorescent AGE products accumulated with chronological age in fully fed and dietary restriction (DR) flies, and in flies where diet was switched, but there was no difference in AGE levels between treatments (20 flies per sample, $n = 3$ samples for each condition). (D) Plotting mortality rate (μx , Fig. 1A) against AGE content revealed that there was a significantly lower ($P < 0.0001$) intercept in the regression line fitted to the DR group when compared with that of the control group indicating that the accumulation of AGEs in DR was delayed when compared with control flies. Once begun, the rate of AGE accumulation in the two groups was identical. Flies switched between DR and fully fed regimes adopted the mortality rate/AGE content relationship of the fully fed controls.

adopted by flies switched from DR to control feed were no different from those of the chronic controls. The poor fit of the regression line for the control to DR switch made statistical assessment of this group unreliable.

Thus, although the absolute AGE signal increased with increasing chronological age in both DR and control flies, the difference in mortality rate, μx , of flies in the two dietary regimes could not be accounted for by a difference in their fluorescent AGE content.

To control for possible alterations in fluorescent AGE composition, we compared spectra obtained from flies aged 7 and 35 days old maintained on both food types. Neither the excitation nor emission maxima changed significantly between young and aged flies (not shown) indicating that composition of these AGEs was not changing with age. Thus, the age-related changes in fluorescence intensity seen in these flies probably represent changes in the quantity of fluorescent material, not changes in the composition of the samples.

Oxidized protein modification in temperature switch flies

The measured protein adducts rose significantly with chronological age in flies maintained at either 18 or 27 °C (Fig. 3A;

$P < 0.001$ in each case except GSA at 18 °C (where $P < 0.01$) and CML at 27 °C (where $P < 0.05$); $n = 3$; one-way ANOVA with Tukey–Kramer *post hoc* test). Additionally, with the exception of CML, at later time points there was significantly more adduct for each protein species in flies maintained at 27 °C than at 18 °C (Fig. 3A). The lack of statistical significance in the CML group may have been due to the relatively small sample size as the trend in these data is clearly similar to all the other measured protein species. In all cases except MDAL ($F = 13.9956$, $DFn = 1$, $DFd = 5$, $P = 0.013$; analysis of covariance) the rate of residue accumulation was not different in flies maintained at different temperatures.

When flies were switched from the 27 °C environment to 18 °C, there was a rapid and complete fall in whole-fly protein adducts in each case (Fig. 3A). In all but AASA the switch in modified protein level was so emphatic that the final values of the switched group were significantly lower than those of the chronic 18 °C group ($P < 0.001$ in all cases). When the reciprocal switch was made, all protein adducts rose until they were indistinguishable from the chronic 27 °C group by Day 34 ($P < 0.05$ in all cases).

The mathematical relationship between mortality rate and protein adduct accrual for all protein adducts was dependent upon environmental temperature (Fig. 3B and Table S3),

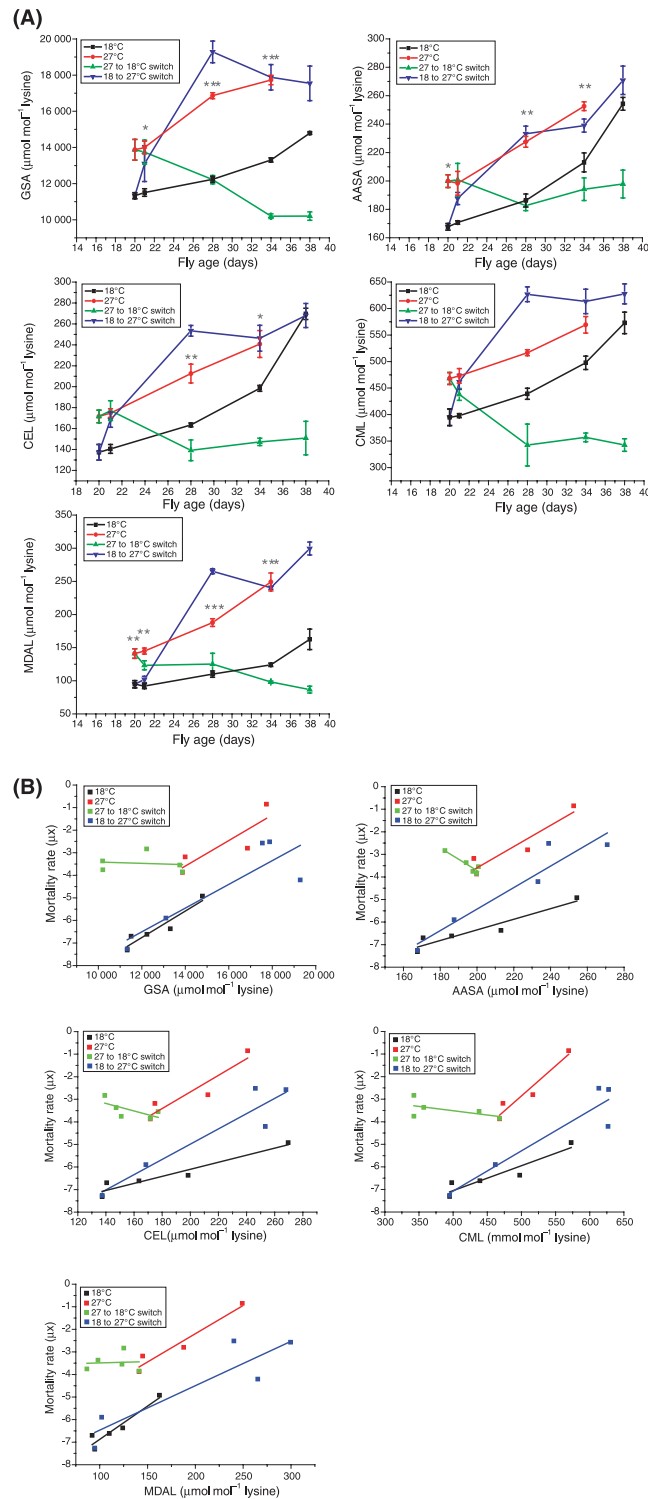


Fig. 3 (A) Effect of temperature on the accumulation of protein adducts. Whole-fly GSA, AASA, CEL, CML and MDAL concentration rose with age in both 18 and 27 °C groups (comparison of first and final day levels by one-way analysis of variance, $n = 3$ in all cases. See text). With the exception of CML (all time points) and the CEL level at day 34, all adduct levels were significantly higher at all time points in the 27 °C group when compared with the 18 °C group. Switching flies between 27 °C and 18 °C at Day 21 induced a complete decrease in the amounts of all measured adducts. The reversal was so emphatic that in all cases the measured amounts of adduct undershot the amounts measured in the chronic 18 °C group. The reciprocal switch induced a rise in all protein adducts in the switched group resulting in no difference between the switched flies and those maintained chronically at 27 °C ($P > 0.05$ in all cases). Error bars represent standard error of the mean ($n = 3$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$. (B) Mathematical relationship between protein adduct accumulation at different temperatures and mortality rate. Plotting protein adduct accrual against mortality rate revealed a different mathematical relationship for each adduct measured (see Table S3).

demonstrating that the rate of protein adduct accrual in the flies did not simply reflect mortality rate (μ x).

Oxidized protein modification in DR and switched flies

There was a significant increase in all the modified protein residues with age on both diets (Fig. 4A; $P < 0.001$ for each residue, one-way ANOVA with Tukey–Kramer Multiple Comparisons *post hoc* test, $n = 5$). Additionally, at later time points, all of the modified protein residues were significantly higher in flies reared on control food than those subjected to DR (Fig. 4A). However, switching the diets of the flies induced a rapid and complete switch of the modified residue content. Flies which had started life on a DR regime and were then switched to control food contained as much GSA, AASA, CEL, CML and MDAL as those which had been maintained chronically on control food ($P > 0.05$, $n = 5$ in all cases) and, with the exception of CEL ($P > 0.05$), each of these values was significantly higher at this time point than those measured in flies chronically fed DR food ($P < 0.001$ in all cases, one-way ANOVA with Tukey–Kramer Multiple Comparisons *post hoc* test, $n = 5$). The reciprocal switch, where flies that started life on control food and were then subjected to DR, was also associated with a reversal of protein adduct content. Levels of all of the measured adducts in the fully fed flies decreased within 5 days to those of the chronic DR group ($P > 0.05$ in each case, $n = 5$).

Plotting the adduct content against mortality rate, μ x, (Fig. 4B) revealed that neither the slope, b , nor the intercept, a , was different between the control and chronic DR groups for any of the measured residues (data not shown). Switching from DR to fully fed reduced the slope, b , but this only reached statistical significance for MDAL ($F = 10.0207$, $DFn = 1$, $DFd = 4$, $P = 0.034$).

Protein adducts in male and female *Drosophila*

Under our laboratory conditions, where the sexes are housed separately after an initial 24-h mating period, female flies lived significantly longer than males when kept on control food at 25 °C (median lifespans of 53 and 45 days, respectively; $P < 0.0001$, Log-Rank test; $n = 527$ females, 603 males). We therefore looked to see whether the oxidized protein adducts differed between the sexes and across the lifespan of each. Similar to the DR and temperature experiments, the protein adducts increased with age in each sex (Fig. 5). The final values (at day 45) were significantly higher than the initial values (at day 7) for each measure; $P < 0.001$ in each case, $n = 5$. Each of the modified protein adducts that we measured was higher in the comparatively shorter-lived male flies at most time points (Fig. 5). There was no difference in the rate of accumulation of GSA, AASA and CEL between the sexes but the initial values were significantly higher in males (GSA: $F = 45.5079$, $DFn = 1$, $DFd = 7$, $P = 0.0003$, AASA: $F = 98.2455$, $DFn = 1$, $DFd = 7$,

$P < 0.0001$; CEL: $F = 23.149$, $DFn = 1$, $DFd = 7$, $P < 0.0019$; analysis of covariance). Male flies accumulated CML and MDAL significantly faster than females ($F = 38.4762$, $DFn = 1$, $DFd = 6$, $P = 0.0008$ and $F = 20.8109$, $DFn = 1$, $DFd = 6$, $P = 0.0038$, respectively).

Discussion

Mortality rates (μ x) in *Drosophila* can be attenuated by maintaining flies at a relatively low environmental temperature (Miquel et al., 1976) or by subjecting them to DR (Chapman & Partridge, 1996). Our results confirm that temperature alters the slope, b , of the mortality trajectory and that flies switched between temperatures permanently bear the mark of their thermal history (Fig. 1A). In contrast, flies fed a DR diet exhibit a delay in the onset of mortality (Fig. 1B), which then increases at the same rate as fully fed controls (Pletcher et al., 2002). Flies switched between diets rapidly and completely adopt the mortality trajectory, b , of flies chronically maintained on the other food type. Increased longevity in flies raised at lower temperatures is due to a reduced rate of accumulation of molecular damage and hence aging (Mair et al., 2003; Partridge et al., 2005) while DR lengthens lifespan in *Drosophila* by reducing the short-term risk of death. Similar effects of DR on mortality patterns have been reported in mice (Weindruch & Walford, 1982; Turturro et al., 2002); however, it remains possible that not all mammals respond to DR in the same way (for a discussion see Masoro, 2009).

Molecular markers that vary with an organism's survival may be regarded as 'biomarkers of aging', accumulating with increasing age and potentially underlying the eventual cause of death. Other markers may track survival but actually reflect risk of dying at any given time due to non age-related causes. The key to differentiating which markers reflect day-to-day risk of death from those which track mortality due to increasing age is whether the markers reverse when life-extending interventions, such as DR, are applied to animals that have begun to age. Although DR extends lifespan it does not reduce aging. DR merely delays the onset of an irreversible process and markers of that irreversible process must therefore be irreversible themselves.

Switching flies between temperatures and diets thus provides a potentially powerful tool to distinguish markers of aging from markers of the risk of dying. Molecular markers that accumulate with chronological age but which then switch when flies are transferred to or from DR cannot, by definition, correspond to the damage that underlies the irreversible process of aging. Instead, they may be markers of the risk of dying. Conversely, markers that accumulate with chronological age at a rate that then decreases when flies are transferred to a cooler environment, without acutely switching to the lower levels seen in chronically cool flies, correspond to the damage that underlies the irreversible process of aging. These are markers of aging and not of the risk of dying. Here we have used this test to examine which of these categories commonly measured fluorescent

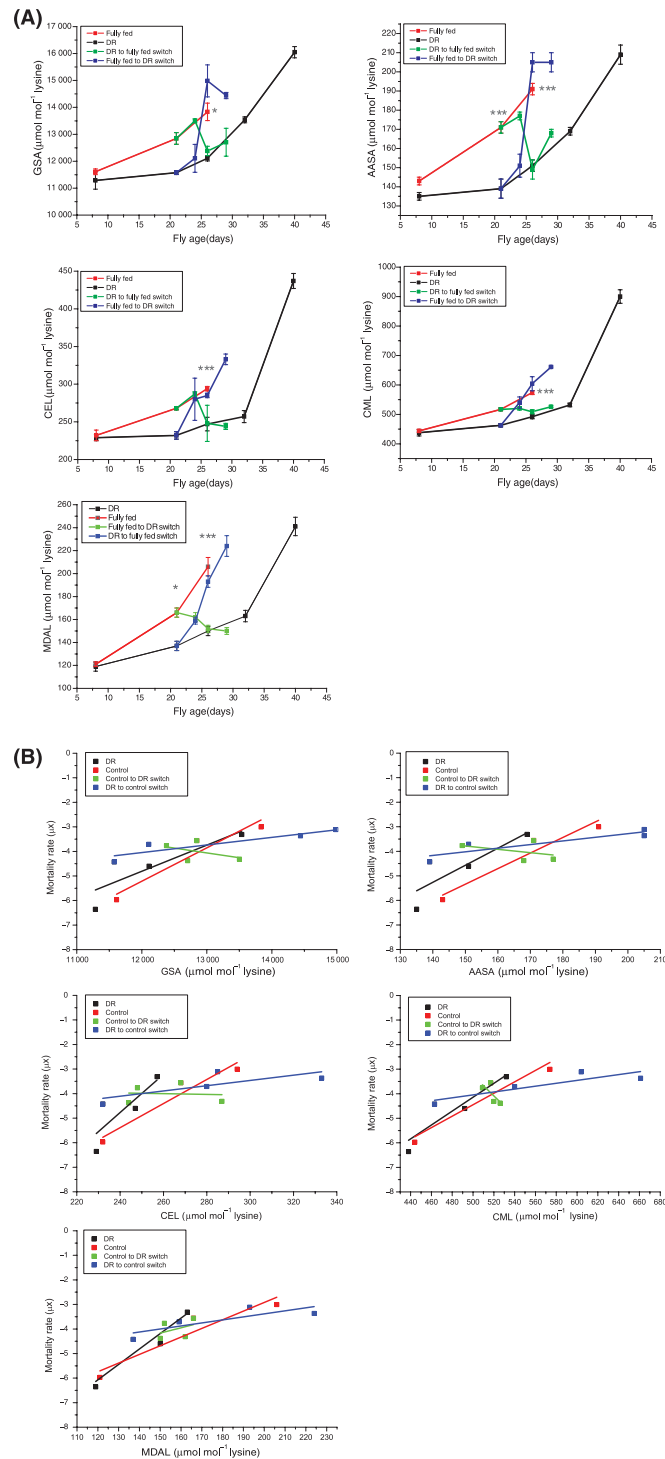


Fig. 4 (A) Effect of diet on the accumulation of protein adducts. Whole fly GSA, AASA, CEL, CML and MDAL concentrations rose with age in both fully fed and dietary restriction (DR) flies (comparison of first and final day levels by one-way analysis of variance, $P < 0.001$, $n = 5$ in all cases. See Table S3). There was no difference in adduct levels between fully fed and DR flies at Day 8 but AASA and MDAL were significantly higher in the fully fed group at Day 21 and by Day 26 all adducts were significantly higher in the fully fed controls when compared with DR flies. Switching fully fed flies to a DR regime induced an immediate and rapid fall in all adduct levels such that levels at Day 26 were indistinguishable from those of the chronic DR flies ($P > 0.05$ in all cases). The converse switch, where DR flies were switched to the control diet, induced an immediate rise in all adducts. In all cases the adduct level of the switched flies was statistically indistinguishable from the chronic DR group by Day 26 ($P > 0.05$ for each assay). Error bars represent standard error of the mean ($n = 5$). * $P < 0.05$; *** $P < 0.001$. (B) Mathematical relationship between protein adduct accumulation and mortality rate in fully fed and DR diets. Neither slope nor intercept was different between fully fed and DR groups when protein adduct accrual was plotted against mortality. Switching from DR to fully fed reduced the slope but this only reached statistical significance for MDAL (see text). There was a further reduction in slope when flies were switched from fully fed to DR but this did not quite reach statistical significance.

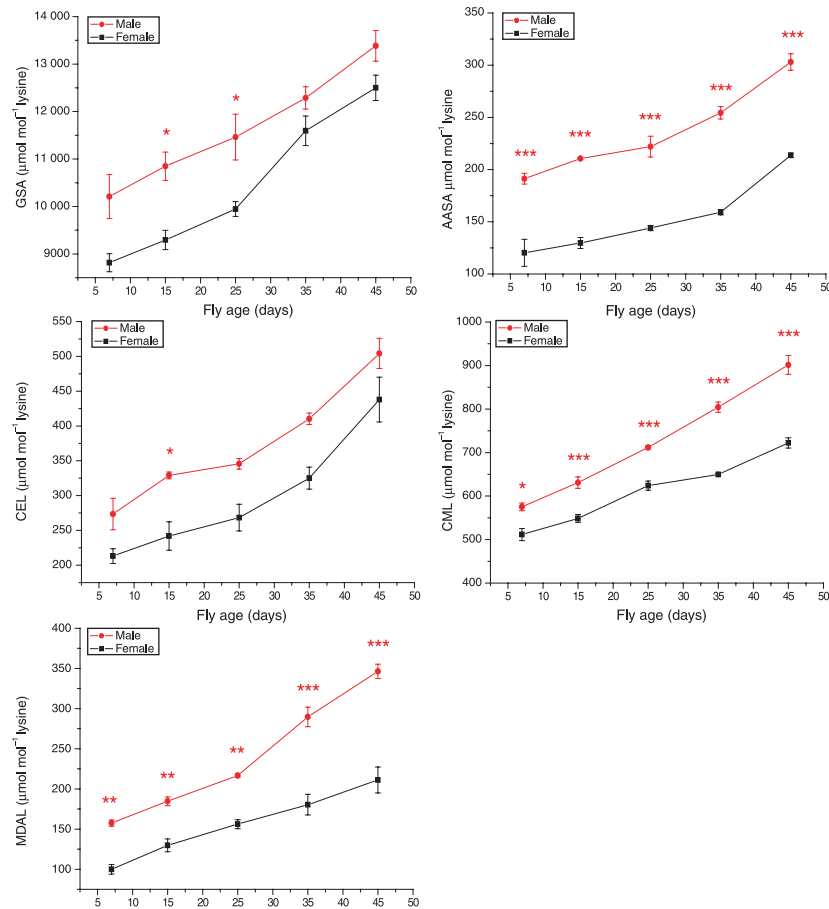


Fig. 5 Effect of sex of flies on the accumulation of protein adducts. Flies were maintained fully fed at 25 °C. All of the measured protein adducts were significantly higher at 45 days when compared with those measured at 7 days for each sex ($P < 0.001$ in each case, $n = 5$ for each measure). Under our laboratory conditions, male flies live shorter lives than females and each protein adduct was higher in males at most time points (one-way ANOVA with Tukey–Kramer Multiple Comparisons *post hoc* test, $n = 5$). * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

protein adducts and oxidized and glycated protein adducts fall into in *Drosophila*.

Consistent with the reports that protein damage increases with aging is our observation that fluorescent AGEs measured in all groups of flies increased with chronological age. Fluorescent AGE accumulation was positively correlated with higher mortality rates in flies maintained at 27 °C, when compared with 18 °C, and the single mathematical relationship between AGE accumulation and mortality rate, μ , in flies at either temperature suggests that the physiological processes underlying the mortality rate in these flies also accounts for the accrual of fluorescent AGEs. This finding is consistent with observations in *C. elegans* (Gerstbrein *et al.*, 2005); however, we also switched flies between temperature and dietary regimes in order to test whether fluorescent AGEs track the aging process or risk of death. The rate of accrual of AGEs in switched flies changed to that characteristic of flies kept permanently at the new temperature, and they therefore permanently carried a level of damage accrued in accordance with their thermal past, suggesting that these fluorescent protein adducts are not removed, at least in the time scale of this experiment. Hence, nonreversible increases

in mortality rate in *Drosophila* are reflected by nonreversible increases in fluorescent AGE accumulation. These observations suggest that fluorescent AGEs are biological markers of aging in *Drosophila*.

The biological fate of fluorescent AGEs remains unclear. While it is possible that these macromolecules accumulate irreversibly and are not removed or modified by any metabolic process, an increasing steady state concentration would result as an imbalance of accrual over removal (Terman & Brunk, 2004). Cell surface scavenger receptors, 'receptors for advanced glycation end-products' (RAGEs), can bind highly modified glycation adducts and oxidized lipoproteins *in vitro* and binding of RAGEs has been shown to precipitate local inflammation (Stehouwer *et al.*, 2002) and protein turnover (Thornalley, 1998; Lu *et al.*, 2004). However, convincing evidence that the *in vitro* properties of AGE receptors reflect *in vivo* action is lacking and the observation that RAGE ligands need to be extensively modified casts doubt on their ability to bind AGEs under physiological conditions (Baynes, 2001; Ahmed & Thornalley, 2007). Whether fluorescent AGEs are causal in determination of death rates or are merely an

accompaniment to aging-related decline awaits further investigation.

In contrast, no differences in fluorescent AGE levels were seen between control and DR groups. Hence, manipulation of the day-to-day risk of death has no effect upon the accumulation of these fluorescent protein derivatives.

Each of the oxidatively derived protein adducts that we studied increased with chronological age in flies maintained on a standard diet at a standard temperature (Figs 3A and 4A). Moreover, all of the protein adduct levels increased with age in both sexes and there were significantly higher levels of most adducts in comparatively shorter-lived males (Fig. 5). These findings are consistent with the idea that these adducts, also, are markers of aging. However, switching flies between dietary regimes produced a rapid switch in all adducts (Fig. 4A), implying that they also are markers of acute risk of death associated with diet.

Remarkably, when flies were switched between thermal regimes, the level of each species of protein adduct also switched, despite the fact that the switched flies' mortality rate (μ) did not switch and only showed a change in slope (*b*). Indeed, the degradation of the modified proteins was so effective that with the switch from high to low temperature the switched flies eventually contained significantly less of the protein adducts than flies that had been maintained at 18 °C all their lives. This observation implies that transfer to a cooler environment can result in upregulated proteolysis and thus removal of some oxidized protein adducts. These data together indicate that, although levels of these adducts increase with age, there is a dynamic equilibrium between protein oxidation and glycation and removal of these adducts through protein turnover. The value of this equilibrium changes with age, and can also be greatly changed acutely, by changes in dietary and thermal regime, and in a way that does not account for differences in mortality rates. These adducts are therefore not causal for aging, or for changes in mortality rate with changes in diet and temperature. This approach has thus been useful for triaging molecular markers into those that are and are not potential candidates for the causes of aging-related decline in function and death.

The effect of temperature on lifespan originally led to the 'rate of living' hypothesis (Pearl, 1928), which proposed that animals have a fixed quota of aerobic energy to dispense in their lives. Subsequently, the free radical theory of aging (Harman, 1956) hypothesized a steady, irreversible accumulation of oxidatively damaged molecules that cause organismal aging. In support, oxidative damage to proteins has been described frequently in models of aging in a number of species ranging from humans (Pansarasa *et al.*, 2000; Traverso *et al.*, 2003; Pamplona *et al.*, 2005) through rodents (Agarwal & Sohal, 1994a; Vittorini *et al.*, 1999; Navarro & Boveris, 2004), flies (Agarwal & Sohal, 1994b; Mockett *et al.*, 1999) and nematodes (Yasuda *et al.*, 1999; Adachi & Ishii, 2000; Yanase *et al.*, 2002), to yeast (Jakubowski *et al.*, 2000; Lee & Park, 2004). Modified proteins have been assayed in interspecific comparisons of aging (Sohal *et al.*, 1995; Portero-Otin *et al.*, 2004) and have been identified in a range of aging tissues from brain (Pamplona *et al.*, 2004, 2005)

to liver (Colantoni *et al.*, 2001), skeletal muscle (Pansarasa *et al.*, 2000) and myocardium (Li *et al.*, 2005). However, proteins modified in different ways by different processes may be turned over by a variety of mechanisms (Dunlop *et al.*, 2009), and some may be only incompletely removed leaving permanent cellular residues (Yin, 1996). It is these last, the irreversibly accumulating damaged protein residues that probably cause the aging process and it is clear that in *Drosophila* fluorescent AGEs are at least part of this irreversible accumulation. Other protein adducts are ultimately removable and thus reflect only the immediate risk of death or disease. The precise molecular consequences of fluorescent AGE accretion await investigation but demonstrably this method of triaging candidate molecules for the aging process can be used to identify other molecular species that underlie the physiology of aging.

Experimental procedures

Fly stocks

The wild-type stock was collected in Dahomey (now Benin) in 1970 and has since been maintained in population cages with overlapping generations. Stocks are maintained at 25 °C, 65% humidity, on a 12-h light:12-h dark cycle and fed standard sugar/yeast medium (Chapman & Partridge, 1996). Eggs were collected from stock cages over an 8-h period and reared on standard sugar/yeast medium at a density of c. 200 per 1/3 pint glass bottle at 25 °C and 65% humidity. Flies enclosing over an 8-h period were collected, transferred to fresh bottles and left to mate for 24 h. Flies were then sorted by sex on CO₂ diffusers and randomly allocated to one of the treatments and the appropriate food nutrient concentration or temperature. Flies were kept in 1/3 pint bottles on 35 mL food throughout. Initial cohort sizes were calculated as the summed death and censor observations over all ages. Flies were transferred onto new food every 2 days and scored for deaths virtually every day.

Food media

Dietary restriction: low nutrient food ('DR') contained: 65 g autolysed yeast powder [B.T.P. Drewitt, London, UK, see (Bass *et al.*, 2007)], 65 g sugar, 16.5 g agar, 30 mL nipagin (100 g L⁻¹), 3 mL propionic acid, 1 L water. Control food ('fully fed') contained: 150 g autolysed yeast powder, 150 g sugar, 20 g agar, 30 mL nipagin (100 g L⁻¹), 3 mL propionic acid, 1 L water. For temperature experiments food contained: 100 g autolysed yeast powder, 100 g sugar, 20 g agar, 30 mL nipagin (100 g L⁻¹), 3 mL propionic acid, 1 L water.

Measurement of fluorescent age products (AGEs)

Fluorescent AGEs were assayed by the method of Oudes *et al.* (1998) with minor modifications. Twenty flies were homogenized in 900 μ L of PBS containing 10 mM EDTANa₂·2H₂O. The homogenate was transferred to a microcentrifuge tube

containing 10 mg of trypsin dissolved in 100 μL of PBS/10 mM EDTA. Following incubation for 24 h at 37 °C, the digested homogenate was centrifuged at 11 000 g for 5 min. The supernatant was spin-filtered (11 000 g for 5 min) through a 0.22 μm cellulose acetate membrane (Costar spin-X[®], Corning, NY, USA). Aliquots of the filtrate (3 \times 200 μL) were transferred to a 96-well plate, and the fluorescence was measured at excitation and emission wavelengths of 365 and 440 nm, respectively. The fluorescence per 20 flies was taken as the mean fluorescence from the triplicate wells.

Measurement of GSA, AASA, CML, CEL and MDAL

Glutamic semialdehyde, AASA samples containing 0.75–1 mg of protein were delipidated using chloroform/methanol and proteins were precipitated by adding 10% trichloroacetic acid and centrifugation. Protein samples were reduced overnight with 500 mM NaBH₄ in 0.2 M borate buffer, pH 9.2, containing one drop of hexanol as an anti-foam reagent. Proteins were then reprecipitated by adding 1 mL of 20% trichloroacetic acid and centrifugation. The following isotopically labelled internal standards were then added: [²H₈]lysine (*d*8-Lys; CDN Isotopes, Pointe-Claire, Quebec, Canada); [²H₄]CML (*d*4-CML), [²H₄]CEL (*d*4-CEL), and [²H₈]MDAL (*d*8-MDAL), prepared as previously described (Fu *et al.*, 1996; Requena *et al.*, 1997); and [²H₅]5-hydroxy-2-aminovaleric acid (for GSA quantification) and [²H₄]6-hydroxy-2-aminocaproic acid (for AASA quantification) (Requena *et al.*, 1997). The samples were hydrolysed at 155 °C for 30 min in 1 mL of 6 N HCl, and then dried *in vacuo*. The *N,O*-trifluoroacetyl methylester derivatives of the protein hydrolysate were prepared as previously described (Requena *et al.*, 1997). Gas chromatography/mass spectrometry (GC/MS) analyses were carried out on a Hewlett-Packard model 6890 gas chromatograph equipped with a 30 m HP-5MS capillary column coupled to a Hewlett-Packard 5973A mass selective detector (Agilent, Barcelona, Spain). Adduct concentration was calculated from standard curves constructed from mixtures of deuterated and nondeuterated standards. Analytes were detected by selected ion-monitoring GC/MS, and the amounts of products were expressed as the ratio of micromole of GSA, AASA, CML, CEL or MDAL/mol of lysine.

Statistics

Mortality (μ_x) was estimated as $\mu_x = -\ln(px)$, where px is the probability of an individual alive at age $x-1$ surviving to age x (Lee, 1992). Log-Rank analysis was performed using JMP 5.0 statistical software (SAS Institute Inc., Cary, NC, USA). Mortality trajectories in all cases were truncated when final sampling for marker analysis was performed. Analysis of mortality trajectories and ordinate intercepts was performed on the linear (post-switch) portion of the mortality data using WinModest software (Pletcher, 1999). Analysis of protein adducts within treatments was performed using GRAPHPAD Prism software (San Diego, CA, USA) and one-way ANOVA with Tukey–Kramer *post hoc* test.

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Author contributions

JJ: Designed experiments, raised and maintained flies, calculated mortalities and statistics, drafted manuscript. AJL: Designed experiments, measured fAGEs, edited manuscript. MP-O: Conducted mass spectrometry, edited manuscript. RP: Conducted mass spectrometry, edited manuscript. TM: Raised and maintained flies, calculated mortalities and statistics. SM: Prepared flies for mass spectrometry, collected data. YD: Raised and maintained flies, collected data. MDB: Supervised project, edited manuscript. LP: Supervised project, edited manuscript.

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Supporting Information

Additional supporting information may be found in the online version of this article:

Table S1 Calculated parameters of a Gompertz mortality model of female flies maintained or switched between different temperatures at Experimental Day 20. Data were fitted to the Gompertz model ($\mu_x = ae^{bx}$) where μ_x represents mortality rate at age x , a is the baseline mortality, and b is the change in mortality rate with age (the slope of the mortality trajectory). Gompertz parameters are shown followed by the upper and lower 95% confidence limits. Mortality modeling was performed on the linear portion of each mortality curve using WinModest software (Pletcher, 1999).

Table S2 Calculated parameters of a Gompertz mortality model of fully-fed male flies, undergoing DR, or switched between food types at Experimental Day 20.

Table S3 Mathematical relationship between mortality rate and protein adduct accrual was different between temperatures. Slope and intercept of regression lines (Figure 3B) for each adduct at each temperature were compared by analysis of covariance.

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