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The functional costs and benefits of dietary restriction in *Drosophila*

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Summary

Dietary restriction (DR) extends lifespan in an impressively wide array of species spanning three eukaryotic kingdoms. In sharp contrast, relatively little is known about the effects of DR on functional senescence, with most of the work having been done on mice and rats. Here we used *Drosophila melanogaster* to test the assumption that lifespan extension through DR slows down age-related functional deterioration. Adult virgin females were kept on one of three diets, with sucrose and yeast concentrations ranging from 7% to 11% to 16% (w/v). Besides age-specific survival and fecundity, we measured starvation resistance, oxidative stress resistance, immunity, and cold-stress resilience at ages 1, 3, 5, and 7 weeks. We confirmed that DR extends lifespan: median lifespans ranged from 38 days (16% diet) to 46 days (11% diet) to 54 days (7% diet). We also confirmed that DR reduces fecundity, although the shortest-lived flies only had the highest fecundity when males were infrequently available. The most striking result was that DR initially increased starvation resistance, but strongly decreased starvation resistance later in life. Generally, the effects of DR varied across traits and were age dependent. We conclude that DR does not universally slow down functional deterioration in *Drosophila*. The effects of DR on physiological function might not be as evolutionarily conserved as its effect on lifespan. Given the age-specific effects of DR on functional state, imposing DR late in life might not provide the same functional benefits as when applied at early ages.

Key words: cost of mating; demographic senescence; dietary restriction; functional senescence; immunity; stress resistance.

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> The common hill-flowers wither, but they blossom again. The laburnum will be as yellow next June as it is now. In a month there will be purple stars on the clematis, and year after year the green night of its leaves will hold its purple stars. But we never get back our youth. The pulse of joy that beats in us at twenty becomes sluggish. Our limbs fail, our senses rot. We degenerate into hideous puppets, haunted by the memory of the passions of which we were too much afraid, and the exquisite temptations that we had not the courage to yield to. Youth! Youth! There is absolutely nothing in the world but youth!
>
> – Oscar Wilde, *The Picture of Dorian Gray* (1891)

Introduction

One may question the self-absorbance and hedonistic lifestyle of Dorian Gray, the main character in Oscar Wilde's novel. But do we not all envy his lack of physical degeneration? While aging is inevitable, not all traits degenerate at the same rate as an organism ages (Herndon *et al*., 2002; Simon *et al*., 2006). Biologists often define senescence as the age-related decline in fitness traits that arises due to internal physiological deterioration (Rose, 1991). Surprisingly, there have been few direct tests of the assumption that demographic and functional senescence are coupled. Studies that have looked at this give equivocal results (Barger *et al*., 2003; see below).

One way to test the relationship between demography and function would be to manipulate lifespan, and then to examine correlated effects on physiological traits. For decades, researchers have used dietary restriction (DR) to successfully extend lifespan in an impressive array of species spanning three eukaryotic kingdoms, including ciliates, yeast, rotifers, nematodes, water fleas, spiders, fruit flies, guppies, mice, rats, hamsters, dogs, and possibly monkeys (Verdonesmith & Enesco, 1982; Weindruch & Walford, 1988; Austad, 1989; Lane *et al*., 2001; Masoro, 2005). In sharp contrast, relatively little is known about the effects of DR on functional senescence, with most of the work having been done on mice and rats (reviewed by Weindruch & Walford, 1988; Masoro, 2002). Studies of rodents have shown that DR can retard age-related deterioration of the nervous system, muscular system, cardiovascular system, endocrine system, reproductive system, and immune system, and that DR retards the onset of age-associated diseases. In yeast, DR retards the age-related increase in generation time (an aging phenotype in yeast) (Jiang *et al*., 2000). In nematodes, DR increases resistance against oxidative and heat stress, although there are methodological problems in applying DR to worms (Houthoofd *et al*., 2005).

Recent studies suggest that extension of lifespan may not necessarily go hand in hand with slower functional senescence.

In rodents, DR appears to have no effect on age-related deterioration in the nervous system of males or on male reproductive senescence, and can delay puberty and retard skeletal growth (Weindruch & Walford, 1988; Masoro, 2002). Other manipulations that extend lifespan, such as mutation or selection, generally increase stress resistance (Grotewiel *et al*., 2005), but the effects on physiology can be absent (Cook-Wiens & Grotewiel, 2002), trait and age specific (Kang *et al*., 2002; Goddeeris *et al*., 2003, 2005; Wessells *et al*., 2004; Broughton *et al*., 2005), and condition dependent (Marden *et al*., 2003). In a study of the long-lived *daf-2* mutant in *Caenorhabditis elegans*, Jenkins *et al*. (2004) showed that mutants may appear healthy in physiological terms, but can quickly go extinct in a competitive environment because of reduced fertility relative to wild-type worms. Reznick *et al*. (2004) found that guppies from a high-predation environment lived longer under laboratory conditions than those from a low-predation environment. However, the long-lived guppies had higher rates of physiological senescence. Taken together, these studies suggest that lifespan extension does not necessarily slow down physiological deterioration of all functional traits.

The fruit fly provides an excellent system in which to explore the consequences of DR on physiological aging. Previous studies have examined the effect of different levels of DR on age-specific mortality and fecundity (Partridge *et al*., 2005b). Building on this previous work, here we study the effects of DR not only on mortality and fecundity, but also on four physiological traits in young and old flies, including resistance to starvation, oxidative challenge, bacterial infection, and cold shock. While each of these traits has been associated directly or indirectly with aging, they are likely to have distinct genetic pathways, and so provide a broad test of whether the ability of DR to increase lifespan extends to functional traits. Our results show that DR in *Drosophila* does not universally slow down functional deterioration, but that its effects are trait and age dependent.

Results

Demographic senescence

Figure 1 illustrates the effect of diet on survival (Fig. 1A) and mortality (Fig. 1B) as a function of age for data from all trials pooled. In Fig. 1C, we plotted for each trial the probability of dying per time step for flies on the 11% and 16% diets, relative to those on the 7% diet. These parameter estimates were determined from the mixed-effects Cox model (Equation 1). Median lifespan ranged from 38 ± 1.2 days on the 16% diet, to 46 ± 2.1 days on the 11% diet, to 54 ± 12 days on the 7% diet (mean ± SE, n = 18 cages per diet) (Fig. 1A). Thus, our results confirm previous findings that DR extends lifespan in flies [$H_0(\beta_2 = 0)$: $z \geq 15.0$, $P < 0.0001$ in all six trials]. As in an experiment like this there is no *ad libitum* control, one could also state that the concentration of sucrose and yeast decreases lifespan and increases the probability per time step of dying (Fig. 1C). Generally, diet had a linear effect on demography, except in fecundity Experiment 1, where the 11% diet was similar to the 7% diet, and

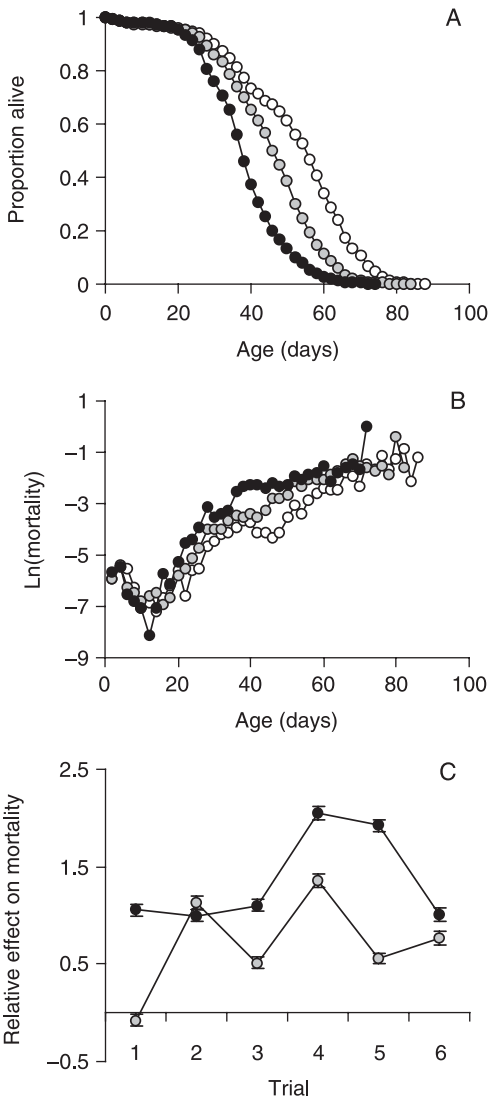

Fig. 1 Effect of diet on demographic senescence. (A) Age-specific survival and (B) age-specific mortality ($n \approx$ 3600 flies per diet). Sucrose and yeast concentration increases from 7% (white symbols) to 11% (gray symbols) to 16% (black symbols). (C) Estimates (± SE) for β, the parameters in the mixed-effects Cox model for the relative effect of the 11% diet (β_1, gray symbols), and 16% diet (β_2, black symbols) on the probability per time step that a fly dies on diet i in cage j. Each trial was a demography experiment run simultaneously with a functional trait: (1) fecundity Experiment 1, (2) fecundity Experiment 2, (3) starvation resistance, (4) oxidative stress resistance, (5) immunity, and (6) cold-stress resilience.

fecundity Experiment 2, where the 11% diet was similar to the 16% diet (Fig. 1C). There is some debate about whether DR extends lifespan through a decrease in baseline mortality or in the rate at which mortality increases with age. In flies, Partridge and colleagues consistently find support for a decrease in baseline mortality (e.g. Pletcher *et al*. 2002; Main *et al*. 2003; Partridge *et al*. 2005c). However, DR has been shown to change the rate parameter in rats (Pletcher *et al*. 2000) and even in flies (Bross *et al*. 2005). An attempt to run a decomposition analysis (Pletcher *et al*., 2000) was unsuccessful, presumably because of cage frailty (see below). Visually, diet lowered the initial mortality rate

more than it decreased the slope of the mortality curve (Fig. 1B), which suggests that its effect was of similar size across all ages.

Reproductive senescence

Figure 2 shows the effect of diet on reproductive output of individual females when held either continuously with three males (Fig. 2A) or intermittently with two males for 1 day per week (Fig. 2B). For both experiments, we plotted the effect of diet on the total number of eggs laid in 33 days (Fig. 2C) and the rate of reproductive senescence (Fig. 2D). Although flies on the 7% diet lived longer (Fig. 1), they laid significantly fewer eggs than flies on either the 11% or 16% diet (Fig. 2C), confirming previous studies. Diet had no significant effect on the rate of reproductive senescence (Fig. 2D), which suggests that the negative effect of DR on fecundity was similar across all ages. The mated flies (Fig. 2) clearly lived shorter than the virgin females that were used to measure demography (Fig. 1). Interestingly, the effect of diet on reproduction depended on the mating conditions. When males were present continuously, reproduction peaked at intermediate concentrations of sucrose and yeast (Fig. 2C). In contrast, when males were present intermittently, fecundity was much higher (Fig. 2C), the rate of reproductive senescence was lower (Fig. 2D), and the 16% diet gave similar fecundity as the 11% diet (Fig. 2C). Note that for Experiment 2, there was also no difference in survival of females maintained on the 11% and 16% diets, but kept as virgins (Fig. 1C). The important point here is that although flies on the 16% diet were short lived, the short lifespan was not simply caused by malnutrition as the 16% diet was a good food source for reproduction. Continuous presence of males, on the other hand, did reduce fecundity, especially under rich food conditions.

Starvation resistance

In 4-day-old flies, DR flies were much more resistant against starvation than control flies [Fig. 3A and Supplementary Fig. S1A; $H_0(\beta_2 = 0)$: $z = 4.21$, $P < 0.0001$]. However, 2 weeks later we found the opposite effect, with DR flies appearing much less resistant to starvation than the controls (Fig. 3A; Supplementary Fig. S1B; $z = -4.62$, $P < 0.0001$). The same negative effect of DR was seen at later ages (Fig. 3A; Supplementary Fig. S1C; $z = -8.71$, $P < 0.0001$; and Supplementary Fig. S1D; $z = -7.47$, $P < 0.0001$). Under starvation conditions, median lifespan of flies kept previously on the 7% diet decreased with age from 66 h (4 days, Fig. S1A) to 40 h (20 days, Fig. S1B) to 36 h (33 days, Fig. S1C) to 24 h (47 days, Fig. S1D) when food deprived, whereas median lifespan of flies kept previously on the 16% diet actually increased with age from 46 h (4 days, Fig. S1A) to 56 h (20 days, Fig. S1B) to 60 h (33 days, Fig. S1C; 47 days, Fig. S1D) when food deprived. During the first two age classes, virtually no flies died in the control treatment (Fig. S1A,B). During the last two age classes, control flies also died in the presence of a sucrose solution. However, the strong negative effect of DR on starvation resistance was already obvious before control flies started dying off [i.e. within 2 days in 33-day-old flies (Fig. S1C) and within 20 h in 47-day-old flies (Fig. S1D)]. Although flies on the 11% diet had an intermediate lifespan (Fig. 1C), diet did not affect starvation resistance in a linear fashion.

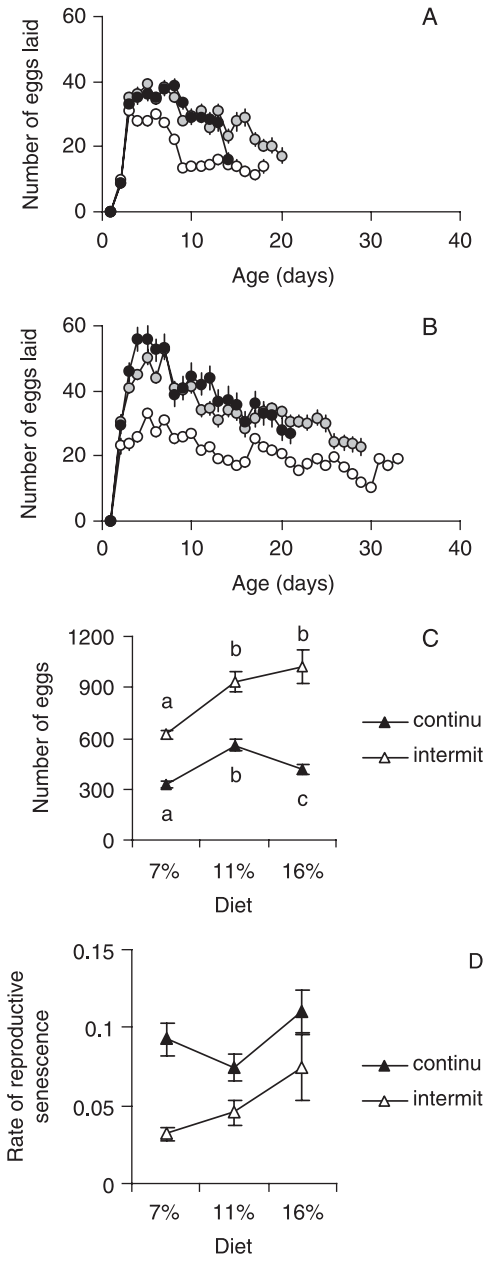

Fig. 2 Effect of diet on reproductive senescence. (A, B) Daily number of eggs laid by individual females (mean ± SE), until less than 20 out of 30 females per diet were alive. Sucrose and yeast concentration increases from 7% (white symbols) to 11% (gray symbols) to 16% (black symbols). (A) Fecundity Experiment 1: each female had continuous access to three males; (B) Fecundity Experiment 2: each female had intermittent access to two young males for 1 day per week. (C) Total number of eggs laid within 33 days (mean ± SE) for Experiments 1 (black symbols) and 2 (white symbols). Within each experiment, means with different lowercase letters are significantly different ($P < 0.05/3$). (D) Rate of reproductive senescence for Experiments 1 (black symbols) and 2 (white symbols). For each individual, we fit the model $Y = ae^{-bX}(1 - e^{-c(X-d)})$, where Y is the number of eggs laid at age X, and a, b, c, and d are parameters. Parameter b represents an individual's rate of reproductive senescence.

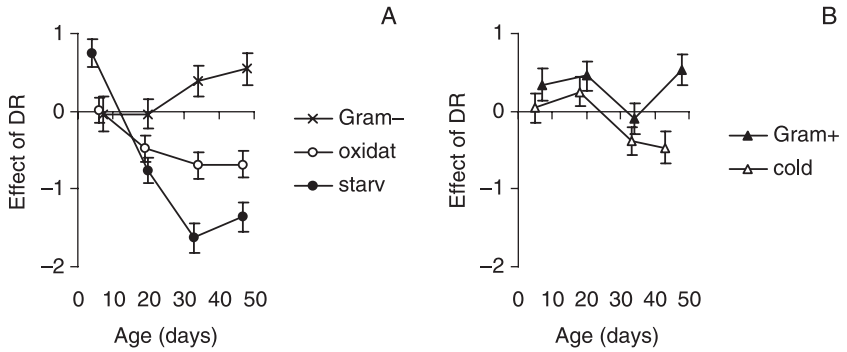

Fig. 3 Age-specific effects of dietary restriction (DR) on five functional traits: (A) starvation resistance (starv), oxidative stress resistance (oxidat), and immunity against *Pseudomonas aeruginosa* (Gram–); (B) immunity against *Lactococcus lactis* (Gram+) and cold-stress resilience (cold). For each age class and functional trait, we plotted the estimate (± SE) for β_2, the parameter in the mixed-effects Cox model (Equation 1) for the relative effect of the 16% diet on the probability per time step that the event occurs on diet *i* in vial *j*. For cold-stress resilience, the event is recovery. Thus, a positive estimate means that flies on the 16% diet had a larger probability per time step of recovering from chill coma than flies on the 7% diet. To interpret the parameter estimate as the effect of DR on cold-stress resilience, we plotted the negative of the estimate for β_2 for recovery. For the other traits, the event is death. Thus, a positive estimate means that flies on the 16% diet had a larger probability per time step of dying than flies on the 7%, i.e. that DR had a positive effect on the trait.

Oxidative stress resistance

In 6-day-old flies, there was no significant effect of diet on oxidative stress resistance [Fig. 3A and Supplementary Fig. S2A; $H_0(\beta_2 = 0)$: $z = 0.081$, $P = 0.94$]. At later age classes, however, DR significantly decreased oxidative stress resistance (Fig. 3 and Supplementary Fig. S2B: $z = -2.885$, $P = 0.0039$; Supplementary Fig. S2C: $z = -4.22$, $P < 0.0001$; Supplementary Fig. S2D: $z = -4.07$, $P < 0.0001$). Survival of control flies kept on a sucrose solution remained fairly high at all age classes. In 34-day-old flies (Fig. S2C), DR had a positive effect on survival of control flies. This reinforces our argument that DR had a negative effect on oxidative stress resistance. This effect was approximately linear.

Immunity

In 7-day-old flies, DR had no significant effect on survival post-infection with the Gram-negative *Pseudomonas aeruginosa* [Fig. 3A and Supplementary Fig. $S3A_1$; $H_0(\beta_2 = 0)$: $z = -0.16$, $P = 0.87$]. Two weeks later, there was still no effect of diet (Fig 3A and Supplementary Fig. $S3B_1$; $z = -0.18$, $P = 0.86$), although the same bacterial concentration resulted in a much steeper decline in survival. Thus, a potential effect of diet could have been masked by the severity of the treatment. This is unlikely the case in the first age class, because a considerable proportion of flies survived the infection. To make the treatment more benign, we lowered the dose to about half the original OD600. In older flies, DR had a positive effect on immunity against *P. aeruginosa* (Fig. 3A and Supplementary Fig. $S3C_1$; $z = 2.03$, $P = 0.042$; Supplementary Fig. $S3D_1$; $z = 2.69$, $P = 0.0072$). This effect appeared earlier and to a larger extent than the effect of diet on sham-infected flies.

Immunity against the Gram-positive *Lactococcus lactis* gave less clear results. Young, 7-day-old flies on 7% food had significantly increased resistance compared with flies on 11% food [Fig. $S3A_2$; $H_0(\beta_1 = 0)$: $z = 2.95$, $P = 0.0032$]. The effect was in the same direction but not significant compared with flies on 16% food [Fig. 3B and Fig. $S3A_2$; $H_0(\beta_2 = 0)$: $z = 1.67$, $P = 0.095$]. Two weeks later, flies on both 7% and 11% food had significantly increased resistance compared with flies on 16% food, although the effect was strongest in flies on 11% food [Fig. 3B and Fig. $S3B_2$; $H_0(\beta_1 = 0)$: $z = -2.28$, $P = 0.023$; $H_0(\beta_2 = 0)$: $z = 2.34$, $P = 0.019$]. In 34-day-old flies, there was no effect of DR on survival post-infection with *L. lactis* [Fig. 3B and Fig. $S3C_2$; $H_0(\beta_2 = 0)$: $z = -0.49$, $P = 0.62$]. In 48-day-old flies, DR significantly increased immunity [Fig. 3B and Fig. $S3D_2$; $H_0(\beta_2 = 0)$: $z = 2.77$, $P = 0.0057$]. In general, DR can have some weak positive effects on immunity against *L. lactis* throughout life.

Cold-stress resilience

In the first two age classes, there was no significant effect of diet on cold-stress resilience [Fig. 3B and Supplementary Fig. S4A; $H_0(\beta_2 = 0)$: $z = -0.23$, $P = 0.82$; Supplementary Fig. S4B: $z = -1.35$, $P = 0.18$]. In the last two age classes, flies kept on 7% diet recovered slightly more slowly from chill coma than flies kept on 16% diet (Fig. 3B and Supplementary Fig. S4C: $z = 2.09$, $P = 0.036$; Supplementary Fig. S4D: $z = 2.29$, $P = 0.022$).

Cage frailty

The effect of diet on demographic senescence was large and always in the same direction, but in each trial we also found significant variation for cage frailty ($\chi^2_{[1]} \geq 7.74$, $P \leq 0.0054$; trial 1: $\hat{\sigma}^2_j = 0.076$; trial 2: $\hat{\sigma}^2_j = 0.143$; trial 3: $\hat{\sigma}^2_j = 0.080$; trial 4: $\hat{\sigma}^2_j = 0.010$; trial 5: $\hat{\sigma}^2_j = 0.057$; trial 6: $\hat{\sigma}^2_j = 0.019$). This means that the cage in which a fly was housed contributed significantly to the variation in the probability per time step of dying. Variation in cage frailty appeared as a temporary leveling off in survival (Fig. 1A) and mortality (Fig. 1B), as some cages went extinct. More importantly, the standard error of the average median lifespan across cages increased dramatically with decreasing

sucrose and yeast concentration. This suggests that cage frailty was diet dependent. Existing statistical packages do not provide a means to test for a diet by frailty interaction in proportional hazards modeling, although this interaction appears qualitatively to be an important component of overall variation.

Discussion

In this study, we set out to test the assumption that life-extending manipulations will also delay age-related functional deterioration. We used DR to slow demographic senescence and measured its age-specific effects on several functional traits. We confirmed previous studies that DR extends lifespan and reduces fecundity. Negative effects of DR on fecundity have been shown in rodents and *Drosophila*, although long-term effects can be positive in female rats (Chippindale *et al*., 1993; Chapman & Partridge, 1996; Masoro, 2002). Our results also confirm earlier studies that showed a negative effect of mating or male harassment on female fecundity (Linder & Rice, 2005), and a diet-dependent cost of mating (Chapman & Partridge, 1996). Mair *et al*. (2004) argued that since DR also extends lifespan in sterile *Drosophila* females, lifespan extension through DR is not simply a result of a re-allocation of resources from reproduction to survival.

In contrast to the effects of DR on survival and reproduction, the consequence of DR on other traits varied not only from trait to trait, but also among ages. The most striking result was that DR initially increased starvation resistance, but dramatically reduced it later in life. We have since qualitatively confirmed this finding in an additional set of experiments using a population of wild-type flies collected from a peach orchard in Watkinsville, Georgia (D. S. Hwangbo, unpublished data). The positive effect of DR on starvation resistance in *Drosophila* has been known for over a decade (Chippindale *et al*., 1993), and increased stress resistance is often used to argue that lifespan extension is not compromised by physiological side effects (Kenyon, 2001). Our data clearly show that the positive effects early in life are offset by negative effects later in life. The initial positive effect of DR on starvation resistance and lipid content is consistent with the hormesis hypothesis (Masoro, 1998), which proposes that DR is a low-intensity stressor that induces investment of resources into stress resistance and survival. DR increases lipid content in young flies (K. J. Min, personal communication). This enables organisms to overcome adverse periods of food scarcity. As food conditions remain poor, however, this increased stress resistance cannot be sustained.

We also found that DR had no effect on oxidative stress resistance early in life, and negative effects at later ages, in contrast to the free radical theory of aging (Harman, 1956; Balaban *et al*., 2005). Although the attenuation of oxidative damage is a popular hypothesis to explain lifespan extension through DR, the evidence for this hypothesis is mixed (Masoro, 2000, 2005; Merry, 2004; Sinclair, 2005). Our data do not support the hypothesis, at least in terms of the ability to resist exogenous sources of oxidative damage. We do not know about rates or levels of endogenous damage. Furthermore, it remains possible that DR flies demonstrate compensatory feeding and therefore ingest more paraquat. There is currently no consensus regarding the effect of DR on feeding behavior. Mair *et al*. (2005) have found that DR does not affect the proportion of time that flies spent on feeding. Others found that DR has no effect on food intake (Bross *et al*., 2005), that DR increases food intake (Carvalho *et al*., 2005), and even that DR decreases food intake (Min & Tatar, 2006). The effect of DR on feeding behavior in the paraquat assay remains to be tested. A useful alternative to the paraquat assay would be to increase oxidative stress by maintaining flies at high oxygen levels. In this case, oxygen exposure would presumably be unaffected by feeding rate.

DR initially had no effect on immunity against *P. aeruginosa*, but positive effects later in life. DR had some weak positive effects on immunity against *L. lactis* throughout life. In *Drosophila*, there are at least two signaling pathways involved in innate immunity. Generally, the Toll pathway is involved in resistance against fungi and Gram-positive bacteria, and the Imd pathway is involved in resistance against Gram-negative bacteria (Lemaitre *et al*., 1997; Tanji & Ip, 2005). If DR affects gene expression differently in these two pathways, this could potentially explain why the effects of DR on immunity against *P. aeruginosa* were different from the effects of DR on immunity against *L. lactis*. Our results support recent studies that suggest a connection between longevity and immunity (Brummel *et al*., 2004; Kurz & Tan, 2004). The positive effects of DR on immunity imply that there may be a causal relationship between lifespan and immunity, or that these two traits share a common regulatory mechanism. On a side note, it remains unclear what the relationship is between survival post-infection, and, for example, expression of antimicrobial peptides (Zerofsky *et al*., 2005).

DR initially had no effect on cold-stress resilience, but weak negative effects later in life. The relationship between lifespan and cold-stress resistance remains unclear. Reduced production of insulin-like peptides extends lifespan but decreases cold-stress resistance (Broughton *et al*., 2005). Overexpression of hsp70 extends lifespan but does not affect cold-stress resistance at any age class (Minois, 2001). Selection for increased lifespan increases cold-stress resistance (Luckinbill, 1998; Bubliy & Loeschcke, 2005), but selection for increased cold-stress resistance either decreases (Anderson *et al*., 2005; Bubliy & Loeschcke, 2005) or does not change lifespan, depending on temperature (Norry & Loeschcke, 2002). Thus, in contrast to the stress theory of aging (Parsons, 1995), it is unlikely that a universal stress-resistance mechanism underlies lifespan extension (Bubliy & Loeschcke, 2005).

Our results provide a challenge for molecular geneticists and physiologists to test hypotheses about the mechanisms that underlie the trait- and age-specific functional responses to DR. This could be achieved by studying the effects of DR in mutants that have reduced or increased resistance against starvation, oxidative stress, pathogens, cold, heat, etc. (as Mair *et al*., 2004, did with fecundity). Several such mutants are already available (De Gregorio *et al*., 2002; Harbison *et al*., 2004; Mourikis *et al*., 2006). Another approach would be to switch animals from one

diet to another and measure correlated responses in functional traits. Switch experiments measuring mortality (Mair *et al*., 2003) and fecundity (Chippindale *et al*., 1993; Good & Tatar, 2001) suggest that DR does not slow down age-related accumulation of damage. Instead, the effects of DR on mortality and fecundity are acute and independent of feeding history. The question of why mortality varies across cages also needs to be addressed, especially because frailty appeared to be diet dependent.

We conclude that DR does not universally slow down functional deterioration in *Drosophila*. Compared with the beneficial effects of DR in rodents, our results suggest that the effects of DR on function might not be as evolutionarily conserved as its effect on lifespan. It has been suggested in the literature that application of DR late in life may provide the same functional benefits as application of DR early in life, since DR has acute effects on mortality (Mair *et al*., 2003). Our results suggest a more cautious interpretation may be called for. Even if feeding history would have no effect on an organism's functional state, functional benefits of DR early in life can be offset by functional costs at later ages.

Experimental procedures

Flies

Drosophila melanogaster (Canton-S) were obtained from Stephen L. Helfand and kept in a large demography cage at room temperature. For each of the six trials (see below), flies were expanded in 177-mL plastic bottles. From these bottles, we collected virgin females that were either placed individually in 25 × 95-mm glass vials (reproduction) or equally distributed among 0.95-L demography cages at 200 flies per cage (other traits). Food was changed every other day. Cages were maintained at 24 °C, about 30% r.h. on a 12:12 h light–dark cycle, and rotated throughout the incubator to prevent confounding effects of microclimate.

Diet

Adult flies had access to one of three diets. The composition of each diet was modified from a recipe provided by Scott D. Pletcher and is given in Table 1. The yeast and sucrose concentration ranged from about 7%, to about 11%, to about 16% (w/v). Water and agar concentrations were adjusted to control the consistency of the food. Active dry yeast was obtained from SciMart (St. Louis, MO, USA).

Demography

For each experiment with a functional trait, we simultaneously collected 1800 virgin females for a demography experiment. These flies were divided into nine cages of 200 flies each. Survival of these flies was scored every other day. Thus, we measured age-specific survival for 6 trials × 3 diets × 3 cages × 200 flies for a total of 10 800 flies.

Table 1 Composition of diets used to manipulate demographic senescence. Each batter was boiled down to 1100 mL. The specific gravity of propionic acid is about 1.0

	Diet					
	7%		11%		16%	
Ingredient	Weight (g)	% (w/v)	Weight (g)	% (w/v)	Weight (g)	% (w/v)
Water	1170	82	1170	74	1170	63
Yeast	78	7.1	120	10.9	180	16.4
Sucrose	78	7.1	120	10.9	180	16.4
Agar	24	2.2	24	2.2	20	1.8
Tegosept (20%)	18	1.6	18	1.6	18	1.6
Propionic acid	3.6	0.3	3.6	0.3	3.6	0.3

Fecundity

Fecundity was measured as daily number of eggs laid by individual females. To measure the effects of diet on fecundity, we set up two experiments. For each experiment we collected 30 females per diet. Females were held individually in vials from the day of eclosion onwards. In the first experiment, females were kept continuously with three males. The second experiment was set up to reduce the effects of harassment of females by males, which can reduce female life expectancy considerably (Partridge *et al*., 2005a). In the second experiment, females had access to two young males of less than 1 week old for the first 2 days and for 1 day per week thereafter. In both trials, flies were transferred daily to new food vials and the number of eggs laid was counted up to 33 days. Thus, we measured age-specific reproduction in 2 trials × 3 diets × 30 flies for a total of 180 flies.

Starvation resistance

Starvation resistance was measured as survival of flies without access to food but with access to water (wet starvation). For each age class, we prepared six vials per diet. Each vial contained one Kimwipe and two circular filter papers. One vial per diet received 2 mL of a 1% sucrose solution (control), and the other five vials received 2 mL of water (starvation treatment). We aspirated 15 flies per diet from the demography cages into each vial without anesthesia. Survival was scored roughly every other hour until virtually all flies were dead. Starvation resistance was measured at 4, 20, 33 and 47 days of age. Thus, we measured starvation resistance for 4 age classes × 3 diets × (1 control vial + 5 treatment vials) × 15 flies for a total of 1080 flies.

Oxidative stress resistance

Oxidative stress resistance was measured as survival of flies with access to paraquat. Paraquat is a compound that generates the free radical superoxide ($O_2^{\cdot-}$), a reactive oxygen species that is

also produced during mitochondrial respiration. Paraquat therefore increases oxidative stress. For each age class, we prepared six vials per diet. Each vial contained one Kimwipe and two circular filter papers. One vial per diet received 2 mL of a 1% sucrose solution (control), and the other five vials received 2 mL of a 1% sucrose solution and 10 mM paraquat (Aldrich, St. Louis, MO, USA) (oxidative stress treatment). We aspirated 15 flies per diet from the demography cages into each vial without anesthesia. Survival was scored roughly every other hour until virtually all flies were dead. Oxidative stress resistance was measured at 6, 19, 34, and 47 days of age. Thus, we measured oxidative stress resistance for 4 age classes × 3 diets × (1 control vial + 5 treatment vials) × 15 flies for a total of 1080 flies.

Immunity

Immunity was measured as survival after septic infection with either *P. aeruginosa* or *L. lactis* bacteria. *Pseudodomonas aeruginosa* is a Gram-negative bacterium and an opportunistic pathogen that occurs in a variety of habitats including soil, water, and plant surfaces. *Lactococcus lactis* is a Gram-positive bacterium that occurs naturally in plant material and insects. Both bacteria species are found in flies derived from natural populations (V. Corby-Harris, unpublished data). Bacteria were obtained from Brian *P. Lazzaro*, who obtained *P. aeruginosa* from the American Type Culture Collection (ATCC) stock center and isolated *L. lactis* from hemolymph and thorax muscle of wild-type flies collected in a fruit orchard (University Park, PA, USA). Both bacteria were cultured in standard nutrient broth. The day before flies were infected, a working stock was incubated at 37 °C, and we aspirated 140 flies per diet from the demography cages into food vials at 10 flies per vial. The day at which flies were infected, the incubated working stock had reached the log phase of growth and was diluted with nutrient broth until an optical density of about 0.2 cm^{-1} at 600 nm was reached using a Helios Delta spectrophotometer (Thermo Electron, Waltham, MA, USA). After the second age class, the bacterial concentration was reduced to an OD600 of about 0.1 cm^{-1}, because flies immunosenesce and the initial concentration was considered too virulent for 5- and 7-week-old flies. For each vial, flies were carbon dioxide anesthetized and poked in the lateral thorax using a minute, stainless steel pin with a 0.1-mm diameter (Fine Science Tools, North Vancouver, BC, Canada) that was dipped in either sterile broth (control) or bacterial culture. Between infections, the pin was sterilized by dipping it in 70% ethanol, wiping it with a Kimwipe, dipping it in another 70% ethanol tube and wiping it again. Out of the 14 vials per diet, we used two vials as control and six vials per bacterial species as treatment. All 42 vials were handled in random order to prevent confounding circadian effects (Lazzaro *et al*., 2004). All flies were infected within about 3 h, after which survival was scored for 48 h. Flies were infected at 7, 20, 34 and 48 days of age. Thus, we measured immunity for 4 age classes × 3 diets × 2 bacterial species × (1 control vial + 6 treatment vials) × 10 flies for a total of 1680 flies.

Cold-stress resilience

Cold-stress resilience was measured as recovery time after induction of chill coma by cold stress (David *et al*., 1998). To prevent confounding effects of handling order, we divided the experiment into three trials per age class and used a Latin square design to randomize diets within trials. For each trial, we aspirated 20 flies per diet from the demography cages into an empty vial. For each trial, one vial per diet was put on melting ice in a cooler, which knocks flies down into a chill coma. After 3 h, vials were returned to room temperature and flies were spread out on filter paper in an open Petri dish. A fly was aspirated into a time- and diet-specific empty vial as soon as it was able to stand on its legs. We changed time-specific vials every minute. Trials were separated by about 30 min. Cold-stress resilience was measured at 5, 18, 33, and 43 days of age. Thus, we measured cold-stress resilience for 4 age classes × 3 diets × 3 trials × 20 flies for a total of 720 flies.

Statistical analysis

All data except reproduction were times to an event. For cold-stress resilience, the event was recovery from chill coma. For the other traits, the event was death. These times to an event were analyzed using a mixed-effects Cox model with cage or vial as random effect (R 2.2.1, macro coxme) (Therneau & Grambsch, 2000; Therneau, 2003):

$$\lambda_{ij}(t) = \lambda_{0j}(t)e^{\beta_1 X_1 + \beta_2 X_2 + Z_j}, \quad (1)$$

where $\lambda_{ij}(t)$ is the probability per time step that the event occurs on diet i in cage or vial j at time t; $\lambda_{0j}(t)$ is the baseline probability per time step; X are indicator variables: $X_1 = 1$ for the 11% diet and 0 otherwise, $X_2 = 1$ for the 16% diet and 0 otherwise; β are parameters for the direction and magnitude of the relative effect of the 11% diet (β_1) and the 16% diet (β_2); and Z_j is the frailty for cage or vial j, which has a normal distribution with mean 0 and variance σ_j^2. Thus, the expected hazard rate in an average cage or vial is $\lambda_0(t)$ for the 7% diet, $\lambda_0(t)e^{\beta_1}$ for the 11% diet, and $\lambda_0(t)e^{\beta_2}$ for the 16% diet. The random effect was tested by a likelihood ratio test, where the test statistic equals twice the difference between the maximum likelihood of a model with random cage or vial effect (R macro coxme) and the maximum likelihood of a model without random cage or vial effect (R macro coxph). This test statistic is χ^2 distributed with one degree of freedom. For Fig. 1, mortality was estimated by $\mu_x \approx -\ln(p_x)$ (Lee, 1992), where p_x is the probability of surviving from $x - 1$ to x.

Analysis of fecundity data was complicated by the fact that repeated-measures data are not independent across ages and count data have non-normal errors. The simplest way to avoid pseudoreplication is to analyze the total number of eggs laid per individual. To test for the age-specific effect of diet, we first fit the following four-parameter model for each individual (R macro nls) (McMillan *et al*., 1970):

$$Y = ae^{-bX}(1 - e^{-c(X-d)}), \quad (2)$$

where Y is the number of eggs laid at age X, a is the intercept parameter that represents the potential maximum number of eggs laid if there was no maturation period [$Y(0)$ if $e^{-c(X-d)} = 0$], b is the slope parameter that represents the rate of reproductive senescence, c is a parameter that represents the maturation rate, and d is the age at first reproduction. If this model did not converge, we fit a generalized linear model with Poisson errors and log link [$e^{-c(X-d)} = 0$; R macro glm], ignoring egg counts during the maturation period. We then tested the effect of diet on the total number of eggs laid and on the estimate for parameter b (the rate of reproductive senescence), using the Wilcoxon rank sum test for pairwise comparisons (R macro wilcox.test). We applied Bonferroni's correction for multiple comparisons by using an adjusted significance level of $\alpha' = 0.05/3$.

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References

Anderson AR, Hoffmann AA, McKechnie SW (2005) Response to selection for rapid chill-coma recovery in *Drosophila melanogaster*: physiology and life-history traits. *Genet. Res.* **85**, 15–22.

Austad SN (1989) Life extension by dietary restriction in the bowl and doily spider, *Frontinella pyramitela*. *Exp. Gerontol.* **24**, 83–92.

Balaban RS, Nemoto S, Finkel T (2005) Mitochondria, oxidants, and aging. *Cell* **120**, 483–495.

Barger JL, Walford RL, Weindruch R (2003) The retardation of aging by caloric restriction: its significance in the transgenic era. *Exp. Gerontol.* **38**, 1343–1351.

Bross TG, Rogina B, Helfand SL (2005) Behavioral, physical, and demographic changes in *Drosophila* populations through dietary restriction. *Aging Cell* **4**, 309–317.

Broughton SJ, Piper MDW, Ikeya T, Bass TM, Jacobson J, Driege Y, Martinez P, Hafen E, Withers DJ, Leevers SJ, Partridge L (2005) Longer lifespan, altered metabolism, and stress resistance in *Drosophila* from ablation of cells making insulin-like ligands. *Proc. Natl Acad. Sci. USA* 102, 3105–3110.

Brummel T, Ching A, Seroude L, Simon AF, Benzer S (2004) *Drosophila* lifespan enhancement by exogenous bacteria. *Proc. Natl Acad. Sci. USA* 101, 12974–12979.

Bubliy OA, Loeschcke V (2005) Correlated responses to selection for stress resistance and longevity in a laboratory population of *Drosophila melanogaster*. *J. Evol. Biol.* **18**, 789–803.

Carvalho GB, Kapahi P, Benzer S (2005) Compensatory ingestion upon dietary restriction in *Drosophila melanogaster*. *Nat. Methods* **2**, 813–815.

Chapman T, Partridge L (1996) Female fitness in *Drosophila melanogaster*: an interaction between the effect of nutrition and of encounter rate with males. *Proc. R. Soc. Lond., B, Biol. Sci.* **263**, 755–759.

Chippindale AK, Leroi AM, Kim SB, Rose MR (1993) Phenotypic plasticity and selection in *Drosophila* life-history evolution. I. Nutrition and the cost of reproduction. *J. Evol. Biol.* **6**, 171–193.

Cook-Wiens E, Grotewiel MS (2002) Dissociation between functional senescence and oxidative stress resistance in *Drosophila*. *Exp. Gerontol.* **37**, 1345–1355.

David RJ, Gibert P, Pla E, Petavy G, Karan D, Moreteau B (1998) Cold stress tolerance in *Drosophila*: analysis of chill coma recovery in *D. melanogaster*. *J. Therm. Biol.* **23**, 291–299.

De Gregorio E, Spellman PT, Tzou P, Rubin GM, Lemaitre B (2002) The Toll and Imd pathways are the major regulators of the immune response in *Drosophila*. *EMBO J.* **21**, 2568–2579.

Gargano JW, Martin I, Bhandari P, Grotewiel MS (2005) Rapid iterative negative geotaxis (RING): a new method for assessing age-related locomotor decline in *Drosophila*. *Exp. Gerontol.* **40**, 386–395.

Goddeeris MM, Cook-Wiens E, Horton WJ, Wolf H, Stoltzfus JR, Borrusch M, Grotewiel MS (2003) Delayed behavioural aging and altered mortality in *Drosophila* beta integrin mutants. *Aging Cell* **2**, 257–264.

Good TP, Tatar M (2001) Age-specific mortality and reproduction respond to adult dietary restriction in *Drosophila melanogaster*. *J. Insect Physiol.* **47**, 1467–1473.

Grotewiel MS, Martin I, Bhandari P, Cook-Wiens E (2005) Functional senescence in *Drosophila melanogaster*. *Ageing Res. Rev.* **4**, 372–397.

Harbison ST, Yamamoto AH, Fanara JJ, Norga KK, Mackay TFC (2004) Quantitative trait loci affecting starvation resistance in *Drosophila melanogaster*. *Genetics* **166**, 1807–1823.

Harman D (1956) Aging: a theory based on free radical and radiation chemistry. *J. Gerontol.* **11**, 298–300.

Herndon LA, Schmeissner PJ, Dudaronek JM, Brown PA, Listner KM, Sakano Y, Paupard MC, Hall DH, Driscoll M (2002) Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. *Nature* **419**, 808–814.

Houthoofd K, Johnson TE, Vanfleteren JR (2005) Dietary restriction in the nematode *Caenorhabditis elegans*. *J. Gerontol. A Biol. Sci. Med. Sci.* **60**, 1125–1131.

Jenkins NL, McColl G, Lithgow GJ (2004) Fitness cost of extended lifespan in *Caenorhabditis elegans*. *Proc. R. Soc. Lond., B, Biol. Sci.* **271**, 2523–2526.

Jiang JC, Jaruga E, Repnevskaya MV, Jazwinski SM (2000) An intervention resembling caloric restriction prolongs life span and retards aging in yeast. *FASEB J.* **14**, 2135–2137.

Kang HL, Benzer S, Min KT (2002) Life extension in *Drosophila* by feeding a drug. *Proc. Natl Acad. Sci. USA* 99, 838–843.

Kenyon C (2001) A conserved regulatory system for aging. *Cell* **105**, 165–168.

Kurz CL, Tan MW (2004) Regulation of aging and innate immunity in *C. elegans*. *Aging Cell* **3**, 185–193.

Lane MA, Black A, Handy A, Tilmont EM, Ingram DK, Roth GS (2001) Caloric restriction in primates. *Ann. N. Y. Acad. Sci.* **928**, 287–295.

Lazzaro BP, Sceurman BK, Clark AG (2004) Genetic basis of natural variation in *D. melanogaster* antibacterial immunity. *Science* **303**, 1873–1876.

Lee ET (1992) *Statistical Methods for Survival Data Analysis*, 2nd edn. New York: Wiley.

Lemaitre B, Reichhart JM, Hoffmann JA (1997) *Drosophila* host defense: differential induction of antimicrobial peptide genes after infection by various classes of microorganisms. *Proc. Natl Acad. Sci. USA* **94**, 14614–14619.

Linder JE, Rice WR (2005) Natural selection and genetic variation for female resistance to harm from males. *J. Evol. Biol.* **18**, 568–575.

Luckinbill LS (1998) Selection for longevity confers resistance to low-temperature stress in *Drosophila melanogaster*. *J. Gerontol. A Biol. Sci. Med. Sci.* **53**, B147–B153.

Mair W, Goymer P, Pletcher SD, Partridge L (2003) Demography of dietary restriction and death in *Drosophila*. *Science* **301**, 1731–1733.

Mair W, Piper MDW, Partridge L (2005) Calories do not explain extension of life span by dietary restriction in *Drosophila*. *PLoS Biol.* **3**, 1305–1311.

Mair W, Sgro CM, Johnson AP, Chapman T, Partridge L (2004) Lifespan extension by dietary restriction in female *Drosophila melanogaster* is not caused by a reduction in vitellogenesis or ovarian activity. *Exp. Gerontol.* **39**, 1011–1019.

Marden JH, Rogina B, Montooth KL, Helfand SL (2003) Conditional tradeoffs between aging and organismal performance of *Indy* long-lived mutant flies. *Proc. Natl Acad Sci USA* **100**, 3369–3373.

Masoro EJ (1998) Hormesis and the antiaging action of dietary restriction. *Exp. Gerontol.* **33**, 61–66.

Masoro EJ (2000) Caloric restriction and aging: an update. *Exp. Gerontol.* **35**, 299–305.

Masoro EJ (2002) *Caloric Restriction: A Key to Understanding and Modulating Aging*, Vol. 1. Amsterdam: Elsevier.

Masoro EJ (2005) Overview of caloric restriction and ageing. *Mech. Ageing Dev.* **126**, 913–922.

McMillan I, Fitz-Earl M, Robson DS (1970) Quantitative genetics of fertility. 1. Lifetime egg production of *Drosophila melanogaster*–Theoretical. *Genetics* **65**, 349–353.

Merry BJ (2004) Oxidative stress and mitochondrial function with aging – the effects of calorie restriction. *Aging Cell* **3**, 7–12.

Min KJ, Tatar M (2006) *Drosophila* diet restriction in practice: do flies consume fewer nutrients? *Mech. Ageing Dev.* **127**, 93–96.

Minois N (2001) Resistance to stress as a function of age in transgenic *Drosophila melanogaster* overexpressing Hsp70. *J. Insect Physiol.* **47**, 1007–1012.

Mourikis P, Hurlbut GD, Artavanis-Tsakonas S (2006) Enigma, a mitochondrial protein affecting lifespan and oxidative stress response in *Drosophila*. *Proc. Natl Acad. Sci. USA* 103, 1307–1312.

Norry FM, Loeschcke VR (2002) Longevity and resistance to cold stress in cold-stress selected lines and their controls in *Drosophila melanogaster*. *J. Evol. Biol.* **15**, 775–783.

Parsons PA (1995) Inherited stress resistance and longevity: a stress theory of ageing. *Heredity* **75**, 216–221.

Partridge L, Gems D, Withers DJ (2005a) Sex and death: What is the connection? *Cell* **120**, 461–472.

Partridge L, Piper MDW, Mair W (2005b) Dietary restriction in *Drosophila*. *Mech. Ageing Dev.* **126**, 938–950.

Partridge L, Pletcher SD, Mair W (2005c) Dietary restriction, mortality trajectories, rich and damage. *Mech. Ageing Dev.* **126**, 35–41.

Pletcher SD, Khazaeli AA, Curtsinger JW (2000) Why do life spans differ? Partitioning mean longevity differences in terms of age-specific mortality parameters. *J. Gerontol. A Biol. Sci. Med. Sci.* **55**, B381–B389.

Pletcher SD, Macdonald SJ, Marguerie R, Certa U, Stearns SC, Goldstein DB, Partridge L (2002) Genome-wide transcript profiles in aging and colonically restricted *Drosophila melanogaster*. *Curr. Biol.* **12**, 712–723.

Reznick DN, Bryant MJ, Roff D, Ghalambor CK, Ghalambor DE (2004) Effect of extrinsic mortality on the evolution of senescence in guppies. *Nature* **431**, 1095–1099.

Rose MR (1991) *Evolutionary Biology of Aging*. New York: Oxford University Press.

Simon AF, Liang DT, Krantz DE (2006) Differential decline in behavioral performance of *Drosophila melanogaster* with age. *Mech. Ageing Dev.* **127**, 647–651.

Sinclair DA (2005) Toward a unified theory of caloric restriction and longevity regulation. *Mech. Ageing Dev.* **126**, 987–1002.

Tanji T, Ip YT (2005) Regulators of the Toll and Imd pathways in the *Drosophila* innate immune response. *Trends Immunol.* **26**, 193–198.

Therneau TM (2003) On mixed-effect Cox models, sparce matrices, and modeling data from large pedigrees. http://mayoresearch.mayo.edu/mayo/research/biostat/upload/kinship.pdf.

Therneau TM, Grambsch PM (2000) *Modeling Survival Data: Extending the Cox Model*. New York: Springer-Verlag.

Verdonesmith C, Enesco HE (1982) The effect of temperature and of dietary restriction on lifespan and reproduction in the rotifer *Asplanchna brightwelli*. *Exp. Gerontol.* **17**, 255–262.

Weindruch R., Walford R. (1988) *The Retardation of Aging and Disease by Dietary Restriction.* Springfield: Thomas.

Wessells RJ, Fitzgerald E, Cypser JR, Tatar M, Bodmer R. (2004) Insulin regulation of heart function in aging fruit flies. *Nat. Genet.* **36**, 1275–1281.

Zerofsky M, Harel E, Silverman N, Tatar M (2005) Aging of the innate immune response in *Drosophila melanogaster*. *Aging Cell* **4**, 103–108.