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Aging in a very short-lived nematode

Michael P. Gardner[a,*], David Gems[b], Mark E. Viney[a]

[a]School of Biological Sciences, University of Bristol, Woodland Road, Bristol, BS8 1UG, UK
[b]Department of Biology, University College London, Gower Street, London, WC1E 6BT, UK

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Abstract

Aging has been characterised in detail in relatively few animal species. Here we describe the aging process in free-living adults of the parasitic nematode *Strongyloides ratti*. We find that the phenomenology of aging in *S. ratti* free-living females, resembles that of the short-lived free-living nematode *Caenorhabditis elegans*, except that it unfolds far more rapidly. The mean (3.0 ± 0.1 days) and maximum (4.5 ± 0.8 days) lifespans of free-living *S. ratti* females are approximately one quarter of equivalent values for *C. elegans*. Demographic senescence (a hallmark of aging) was observed in free-living *S. ratti*, with a mortality rate doubling time of 0.8 ± 0.1 days (females), compared with 2.0 ± 0.3 in *C. elegans*. *S. ratti* lifetime fertility and lifespan were affected by temperature, and there is an age-related decline in feeding rate and movement, similar to *C. elegans*, but occurring more quickly. Gut autofluorescence (lipofuscin) also increased with age in *S. ratti* free-living females, as in aging *C. elegans*. These findings show that the extreme brevity of life in free-living *S. ratti* adults, the shortest-lived nematode described to date, is the consequence of rapid aging, rather than some other, more rapid and catastrophic life-shortening pathology.
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1. Introduction

In this study, we describe the first stage of a full characterisation of aging in the nematode *Strongyloides ratti*. By studying this organism, insight may be gained into the extent of variation in the lifespan of animal species and of phenotypic plasticity in aging and into the evolution of longevity and aging.

The life-cycle of *S. ratti* contains both free-living and parasitic stages (Fig. 1). The parasitic stage is female only and lives in the gut of its rat host and reproduces by mitotic parthenogenesis (Viney, 1994), passing eggs via the faeces into the external environment. The eggs can develop either directly into infective third stage larvae (iL3s) or into free-living adult females and males (Fig. 1). These adults mate by conventional sexual reproduction and produce progeny which develop into iL3s and infect new hosts (Viney et al., 1993). Anecdotal observations have noted a large difference in the lifespan potential of the adult free-living and parasitic female morphs (approximately 1 week and 1 year, respectively) (Viney, 1994; Gemmill et al., 1997). Given that these two adult morphs are genetically identical (Viney, 1994), such a difference in survival is potentially a consequence of differences in gene expression alone.

Why study aging in *S. ratti*? Comparative studies of aging in different animal species can provide basic information about the forms of aging that are biologically possible. For example, studies of the Cnidarians *Hydra vulgaris* (Martínez, 1998) and *Cereus pedunculatus* (Comfort, 1979) suggest that neither of these species senesce. Among animals that age, there is great variation in longevity (Finch, 1990). For example, among mammals, maximum lifespans range from around 3 years in the mouse, *Mus musculus*, to an estimated 211 years in the bowhead whale, *Balaena mysticetus* (George et al., 1999). More striking variation in aging exists in invertebrate taxa. For example, estimates of maximum lifespans in nematode

* Corresponding author. Tel.: +44-117-928-7470; fax: +44-117-925-7374.
E-mail address: mike.gardner@bristol.ac.uk (M.P. Gardner).

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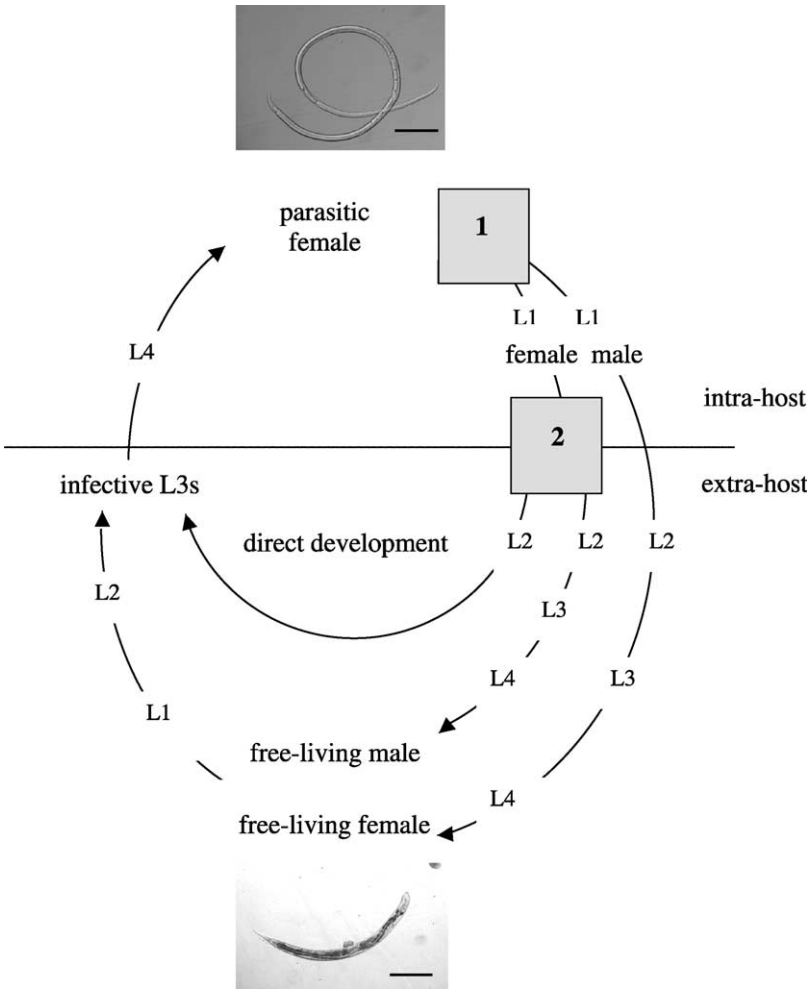


Fig. 1. Life-cycle of *Strongyloides ratti* with two discrete developmental switches, shown as grey boxes: (1) a sex determination event; (2) a female only developmental switch. Taken from Harvey et al., 2000. Also included are larval stages L1–L4. The life-cycle contains both parasitic and free-living generations; an adult parasitic female (top) and free-living adult female (bottom). Bars = 200 μM.

species range from 7.9 days in the terrestrial nematode *Mesodiplogaster biformis* (Sohlenius, 1969) to 20 years in the parasitic nematode *Loa loa* (Gems, 2001).

Studies that relate species' ecology to aging have also been used to interrogate the evolutionary theory of aging. The kernel of this theory is that due to the forces of extrinsic mortality (e.g. starvation, predation, disease), the distribution of the probability of reproducing will be skewed to earlier ages, even in a theoretically non-aging organism. A predicted consequence of this is that the force of natural selection against alleles with late-acting deleterious effects will be less than those with earlier-acting effects (Williams, 1957). The resulting accumulation of alleles with late-acting, deleterious effects (and perhaps early acting, fitness-enhancing effects) is the cause of aging. From this, the maximum lifespan which any species has evolved is predicted to have some correspondence with the level of extrinsic mortality typically experienced by that species in its environment (Edney and Gill, 1968).

This prediction has been supported by a growing number of comparative studies of aging. For example, comparisons between rodents and similar sized bats show increased lifespan in the latter (Austad and Fischer, 1991); the increased longevity of bats is believed to be the consequence of the reduction in extrinsic mortality resulting from the capacity for flight, which reduces predation (Wilkinson and South, 2002). Similarly, Virginia opossums (*Didelphis virginiana*) from an island population with reduced exposure to predators, exhibited delayed aging compared with a control, mainland population (Austad, 1993). In eusocial organisms, lower mortality rates experienced by certain morphs results in evolution of greater longevity in those morphs, e.g. in honeybees (Page and Peng, 2001), ants (Chapuisat and Keller, 2002) and naked mole rats (Sherman and Jarvis, 2002).

Many animal species also show phenotypic plasticity in aging, of which two types may be distinguished. The first is the relatively limited response to a changing environment by a given stage in the life-cycle. An example of this is experimental caloric restriction, which extends lifespan in numerous species, including the nematode *C. elegans* (Klass, 1977; Lakowski and Hekimi, 1998), the fruitfly

Drosophila melanogaster (Nusbaum and Rose, 1999), rodents (McCay et al., 1935) and, probably, primates (Roth et al., 2002). Another example is the effect of diapause on aging; for example, in the Turkish hamster, *Mesocricetus brandti*, hibernating animals lived significantly longer than those not hibernating (Lyman et al., 1981).

A more dramatic form of phenotypic plasticity in aging is associated with the development of different morphs within a given life-cycle. For example, in *C. elegans*, there is a facultative diapausal form of the third stage larva, the dauer larva, which can survive for up to 3 months (Klass and Hirsh, 1976). This compares to a two-to-three week lifespan in *C. elegans* adults (Klass, 1977). Striking variation between lifespan in adult morphs also occurs in social insects (Chapuisat and Keller, 2002). Understanding the evolutionary and physiological determinants of such plasticity can provide insight into the biology of aging.

The aims of the present study are to characterise aging in free-living adult females and males of *S. ratti* and to determine the factors that affect their lifespan. In particular, a major question is whether the reported short lifespan of *Strongyloides* spp. and of related species such as *Rhabdias bufonis* (Yamada et al., 1991; Spieler and Schierenberg, 1995) is actually a consequence of rapid aging, rather than extrinsic, environmental causes, or some form of major defect leading to death. In *C. elegans*, lifespan is reduced by the *Escherichia coli* bacteria on which they are usually cultured (Gems and Riddle, 2000; Garigan et al., 2002; Garsin et al., 2003), by higher temperatures (Klass, 1977), mating between the sexes (Gems and Riddle, 1996) and attempted mating between males (Gems and Riddle, 2000). We have tested the effect of these factors on lifespan in *S. ratti*, and in so doing optimised culture conditions for survival, in order to maximise the contribution of intrinsic senescence to lifespan and to minimise that of deleterious environmental factors. We then asked whether free-living *S. ratti* adults cultured under optimised conditions exhibit demographic aging, i.e. an age-dependent mortality rate acceleration, a hallmark of aging (Wachter and Finch, 1997). In *C. elegans*, there is an increase in autofluorescence with age (Davis et al., 1982; Garigan et al., 2002), similar to accumulation of age-pigment (lipofuscin) seen during mammalian aging. We have tested whether this occurs in free-living *S. ratti* adults.

We find that with culture conditions optimised for survival, *S. ratti* free-living adults exhibit a Gompertzian acceleration of age-specific mortality. By comparison with *C. elegans*, this acceleration was more rapid (indicating faster aging) and the initial mortality rate (IMR) was greater (implying greater frailty). Thus, the *S. ratti* free-living morph is the shortest lived and most rapid-aging nematode described to date.

2. Materials and methods

2.1. Nematodes and bacteria

The *S. ratti* isofemale line, ED321 Heterogonic (Viney, 1996), was maintained by serial passage in female Wistar rats (B and K Universal) or female nude rats (RNU-*rnu*) (Harlan, UK). Food and water was provided *ad libitum* and nude rats were maintained on water containing 0.01% (v/v) Baytril (Bayer, UK), a broad spectrum antimicrobial agent. Infections were initiated by sub-cutaneous injection of 1000 infective third stage larvae (iL3s) and faecal material was collected from rats overnight in grid-bottomed cages and was cultured and maintained at 19 °C using standard methods (Viney, 1994). *C. elegans* wild-type laboratory strain, Bristol (N2), *E. coli* OP50 and streptomycin resistant *E. coli* OP50 (*E. coli* SR) bacterial food sources were obtained from the Caenorhabditis Genetics Center (CGC). *C. elegans* was maintained on NGM agar at 19 °C and seeded with *E. coli* OP50 as a food source (Sulston and Hodgkin, 1988), unless otherwise stated.

2.2. Culture conditions for free-living S. ratti

L4 female and male *S. ratti* worms were taken from 2-day-old faecal cultures of infected rat faeces, cleaned to remove associated bacteria by repeated washing in sterile distilled water and cultured on NGM plates under optimised culture conditions (400 μg/ml streptomycin, *E. coli* SR), unless otherwise stated. Lifespan was measured as described for *C. elegans* (Klass, 1977). Briefly, worms were transferred daily to fresh plates and the number of survivors scored. Cultures were maintained at 19 °C and 10 L4 *S. ratti* females were placed on each plate (100 worms per treatment), unless otherwise stated. Kaplan–Meier analysis was used to compare mean lifespan between treatments using a log-rank χ^2 test.

The lifespan of virgin female *S. ratti* under optimised culture conditions (400 μg/ml streptomycin, *E. coli* SR) was compared with lifespans on non-proliferating bacteria. Non-proliferating bacteria were produced, as previously described (Garigan et al., 2002). Briefly, *E. coli* OP50 was seeded onto NGM plates and allowed to grow for 2 days at room temperature before the antibiotics were added; 20 μg/ml kanamycin or 200 μg/ml streptomycin as bactericidal antibiotics and 12.5 μg/ml tetracycline as a bacteriostatic antibiotic. Cultures of *Bacillus* spp. or *Streptococcus faecalis* isolated from rat faeces were identified using Macconkey and blood agar indicator plates, and Gram staining (Cowan et al., 1993). Nematodes were maintained in 60 mm Petri dishes, except for single worms in population density measurements, which were maintained in 35 mm dishes. All statistical analyses were performed using the JMP statistical analysis package (version 5.0, SAS Institute, Inc, Cary, NC, USA), with level of significance tested at a level $P<0.05$.

2.3. Analysis of age-specific mortality

Survival of virgin free-living *S. ratti* females was measured under optimised culture conditions (400 μg/ml streptomycin, *E. coli* SR) and a temperature of 25 °C (optimal for fertility). *C. elegans* survival was measured under two conditions: (i) *S. ratti* optimised conditions to which was added 60 μl of the supernatant in which the *S. ratti* worms were washed, (the latter introduced low levels of contaminating microbes, assumed to be present under optimised *S. ratti* culture conditions, allowing comparison of survival of *S. ratti* and *C. elegans* under near identical culture conditions); (ii) monoxenic conditions consisting of NGM plates with *E. coli* SR but with no added supernatant. 25 worms per plate and 500 worms per treatment were used, and mortality scored approximately every 8 h for *S. ratti* and every 12 h for *C. elegans*, until all animals were dead. Mortality data were fitted to the Gompertz equation, $m(t)=Ae^{\alpha t}$, where $m(t)$ is the mortality rate at time t, A is the IMR and α is the Gompertz exponential function (Finch, 1990). The occurrence of an age-specific mortality rate acceleration is a definitive feature of aging (Finch, 1990). From plots of ln (mortality rate) against age, we calculated A, the IMR, the mortality rate doubling time (MRDT) and estimated median (time for 50% survival) and maximum lifespans and their standard errors. Mortality parameters for *S. ratti* and *C. elegans* were compared using Student's *t*-tests.

2.4. Fecundity analysis

Free-living *S. ratti* females and males were maintained under optimised culture conditions (400 μg/ml streptomycin, *E. coli* SR). Gravid free-living females were allowed to lay eggs, and transferred daily to fresh plates and the number of eggs were recorded. These were allowed to hatch over a 2-day-period and the number of resulting larvae (i.e. *viable* egg production) counted, and daily and total fecundities calculated over a range of temperatures (4, 10, 15, 19, 25, 30 and 37 °C). We tested whether fecundity in female *S. ratti* was limited by availability of sperm, using two mating regimes. Firstly, 10 virgin free-living male and female *S. ratti* were allowed to mate overnight on plates and the males were removed the next day; secondly, 20 virgin free-living female *S. ratti* and 10 virgin male *S. ratti* were allowed to mate overnight and the old males removed and 10 new ones added daily. Non-parametric Wilcoxon analyses (using χ^2 test) were used to compare life-time fecundity in the two treatments and life-time fecundity across temperature. The effect of age on daily fecundity was tested using linear regression analysis.

2.5. Pharyngeal pumping rate

This was measured during aging in free-living *S. ratti* females as for *C. elegans* (Kenyon et al., 1993; Gems et al., 1998). A single pump was scored as a complete backward movement of the terminal bulb of the pharynx, viewed at 40× magnification, using a Zeiss Stemi 2000-C stereomicroscope (Keane and Avery, 2003). Free-living *S. ratti* females were maintained under optimised culture conditions (25 °C) and each day over a 4-day-period, 20 randomly selected nematodes were placed on separate plates, allowed to acclimatise for one minute and then for each pumping worm, the number of pumps in two, one minute intervals was counted. From this, the average pumping rate and the percentage of animals pumping each day was calculated. If no pumping was seen in either one minute scoring interval, the worm was scored as non-pumping. The effect of age on the mean pharyngeal pumping rate and on the percentage of animals pumping was tested using linear regression analysis.

2.6. Movement

L4 *S ratti* females were placed on each of eight plates and individual L4 *S. ratti* females were placed on 40 plates and maintained under optimised culture conditions (25 °C). The movement of grouped and individual free-living females was observed daily and classified as for *C. elegans* (Herndon et al., 2002). Class A worms move voluntarily and leave sinusoidal tracks, class B worms only move when prodded and leave non-sinusoidal tracks and class C worms do not move forward or backward even when prodded. Non-parametric Wilcoxon analyses (using χ^2 test) were used to compare percentages of class A, B and C of grouped worms on each day, to test whether there was an age-related decline in movement of *S. ratti*. The analysis of individual worms tested whether there was variation in the timing of any age-related decline in movement.

2.7. Autofluorescence

Daily microfluorometric measurements of free-living *S. ratti* females aged 1–6 days (19 °C) were taken using a Leica DMRXA2 microscope using either a narrowband DAPI filter set (excitation: 340–380 nm), or a GFP filter set (excitation: 450–490 nm). In aqueous *C. elegans* extracts there is a peak of fluorescence with an excitatory wavelength range 330–350 nm, and an emission peak at 430–460 nm (32). This blue fluorescence, which increases in intensity with age, is visualised using the DAPI filter set. The GFP filter was employed since use of a similar filter set revealed age increases in autofluorescence in *C. elegans* (Garigan et al., 2002). Images at 100× magnification were captured using a Hamamatsu OrcaER digital camera, and pixel density of selected regions of images of fluorescent nematodes measured using the Openlab 3.1.4 (Improvision, www.improvision.com) image analysis software. Specifically, for each nematode,

the most anterior region of the gut in which well-focused, punctate autofluorescent granules were visible was selected and images captured with the same exposure, which avoid pixel saturation. Sometimes distension of the anterior intestine led to diffuse fluorescence, in which case a more posterior region of intestine was selected. In some 6-day-old animals in which the intestine was not clearly distinct, these were excluded from study. For both DAPI and GFP filters, autofluorescence was measured in ≥ 16 animals apart from day six, when 12 animals were used. The effect of age on fluorescence was tested using linear regression analysis.

2.8. Microscopic examination of aging nematodes

Appearance of major anatomical features was compared in one and 4-day-old *S. ratti* females using Nomarski (differential interference contrast) microscopy, with magnification up to 1000×, using standard *C. elegans* methods (Schnabel, 1999).

3. Results

3.1. Optimisation of culture conditions for S. ratti survival

In contrast to *C. elegans*, it is not possible to prepare cultures of *S. ratti* that are monoxenic (i.e. with *E. coli* alone) since larvae are isolated directly from rodent faeces. To attempt to minimise microbial contamination from the latter (which might otherwise reduce survival), *S. ratti* was cultured on agar plates with streptomycin-resistant *E. coli* OP50 (*E. coli* SR) and a range of concentrations of streptomycin (50, 200, 400, 700 or 1000 μg/ml). Under these conditions, visible contamination by other microbes was reduced but not prevented. Maximum mean life-span was 4.2 ± 0.2 days at 400 μg/ml streptomycin which was significantly greater than on *E. coli* OP50 without antibiotic (mean lifespan: 3.7 ± 0.2 days; $\chi^2 = 11.4$, $P = 0.0007$; Fig. 2A).

In *C. elegans*, prevention of bacterial proliferation leads to extended lifespan, showing that lifespan is shortened by the *E. coli* food source (Garigan et al., 2002). We explored whether this might be the case in *S. ratti*. However, mean lifespan of virgin females under optimised conditions (400 μg/ml streptomycin, *E. coli* SR) was greater (4.1 ± 0.2 days) than on non-resistant *E. coli* to which had been added 200 μg/ml streptomycin (2.4 ± 0.1 days; $\chi^2 = 50.7$, $P < 0.0001$), 20 μg/ml kanamycin (2.9 ± 0.2 days; $\chi^2 = 24.6$, $P < 0.0001$) or 12.5 μg/ml tetracyclin (3.2 ± 0.1 days; $\chi^2 = 17.6$, $P < 0.0001$).

To explore whether *S. ratti* survives better if cultured on bacteria found in their natural environment, it was cultured on either of two bacterial isolates from rat faeces, identified as *Bacillus* spp. and *S. faecalis*. Mean lifespan proved to be longer on *E. coli* SR with 400 μg/ml streptomycin (4.2 ± 0.2 days) than on either *Bacillus* spp. (1.7 ± 0.1 days; $\chi^2 = 89.1$, $P < 0.0001$) or *S. faecalis* (1.5 ± 0.1 days; $\chi^2 = 96.0$, $P < 0.0001$).

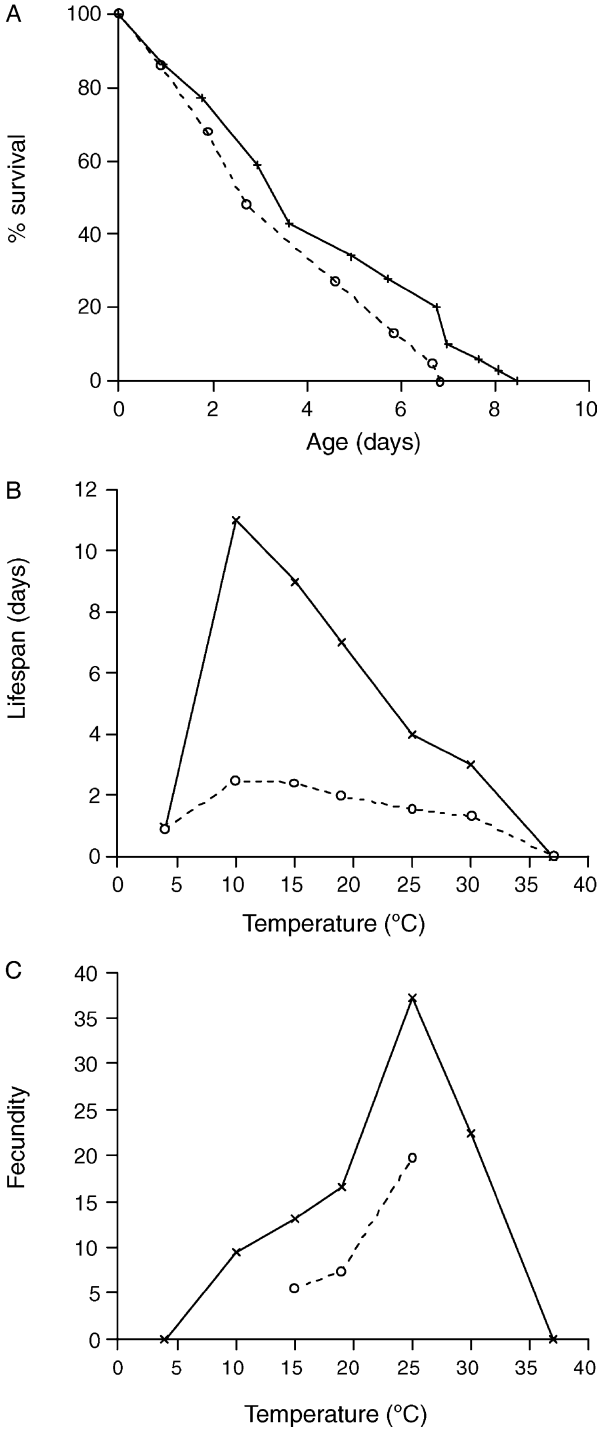


Fig. 2. Factors affecting *S. ratti* lifespan. (A) Survivorship of virgin free-living females under optimised culture conditions (400 mg/μl streptomycin and *E. coli* SR) (x, —) and on *E. coli* OP50 (o, - - -). (B) Effects of temperature on mean (o, - - -) and maximum (x, —) lifespan of virgin free-living females. *S. ratti* adults die within 24 h at 37 °C. (C) Effect of temperature on lifetime fecundity per animal of *S. ratti* females, mating on day one only (o, - - -) and mating throughout life (x, —).

Table 1
The effect of sex-ratio on mean lifespan of female and male *S. ratti* (19 °C)

Animals per plate		Male/ female ratio	Mean lifespan (days±SE)	
Males	Females		Females	Males
0	100	0	2.83±0.19	–
20	80	0.25	2.13±0.22	2.89±0.42
40	60	0.67	2.52±0.28	2.33±0.21
60	40	1.5	3.13±0.39	1.79±0.17
80	20	4.0	3.18±0.40	1.69±0.13

3.2. Environmental and behavioural determinants of fecundity and lifespan

To test the effect of sex-ratio on free-living male and female *S. ratti* lifespan, L4 worms of each sex were maintained on plates (100 animals/treatment, 25 animals/plate) at different male: female ratios. As the ratio of males to females increased, there was no affect on female lifespan (Spearman's rank correlation, $r=0.7$, $P=0.19$) but male lifespan decreased (Spearman's rank correlation, $r=-1.0$, $P<0.0001$) (Table 1), suggesting that mating aids survival in *S. ratti* males.

To test the effect of population density on lifespan, virgin free-living male *S. ratti* were maintained at densities of 1, 25, 50 and 100 animals/plate and virgin free-living females at densities of 1, 20, 50 and 100 animals/plate. For *C. elegans*, lifespan is reduced by increased population density in males but not hermaphrodites (Gems and Riddle, 2000). In *S. ratti,* female lifespan did not differ between female densities of 1 and 50 animals per plate ($\chi^2=0.57$, $P=0.43$), but it did between densities of 1 and 100 animals per plate (1 vs. 100: ($\chi^2=45.2$, $P<0.0001$; Table 2). Likewise, male lifespan did not differ between densities of 1 and 50 animals per plate ($\chi^2=3.6$, $P=0.06$), but it did between densities of 1 and 100 animals per plate (1 vs. 100: $\chi^2=9.6$, $P=0.002$; Table 2).

To test the effect of temperature on lifespan of virgin free-living *S. ratti* females, lifespan was measured at 4, 10, 15, 19, 25, 30 and 37 °C, with 20 worms per plate and 100 worms per treatment. There was a significant reduction in both mean and maximum lifespan with increasing temperature over the range 10–30 °C (mean: $F_{1,3}=78.1$, $P=0.003$; maximum: $F_{1,3}=196.7$, $P=0.0008$; Fig. 2B).

Lifetime fecundity was higher when the females were supplied with fresh, virgin males each day at 15, 19 and 25 °C ($\chi^2=4.9$, $P=0.028$; $\chi^2=6.9$, $P=0.009$; $\chi^2=4.8$, $P=0.028$, respectively) (Fig. 2C). Daily fecundity declines with age at both 19 and 25 °C ($F_{1,4}=15.5$, $P=0.017$; $F_{1,2}=35.0$, $P=0.027$, respectively) and lifetime fecundity varies significantly with temperature ($\chi^2=18.9$, $P<0.0001$; Fig. 2C) and at 25 °C was significantly greater than at any other temperature (data not shown). Therefore, by this criterion, 25 °C is closest to the optimal temperature for fitness. However, under these conditions, the maximum lifetime fecundity was less than 40 progeny per female, compared with over 1400 in *C. elegans* (Hodgkin and Barnes, 1991).

Interestingly, there was a decrease in post-reproductive lifespan with increasing temperature. As a percentage of maximum lifespan, this was 50% at 10 °C, 56% at 15 °C, 25% at 19 °C, 0% at 25 °C and 30 °C. This contrasts with *C. elegans*, where there is a large (e.g. 60% at 20 °C) post-reproductive lifespan that is not affected by temperature (Klass, 1977; Gems, 2002).

Table 2
Effect of density on mean lifespan of virgin *S. ratti* (19 °C)

Population density (animals/plate)	Mean lifespan (days±SE)	
	Females	Males
1	3.09±0.21	2.07±0.15
20, 25[a]	3.11±0.17	1.91±0.09
50	3.14±0.19	2.02±0.09
100	1.67±0.09	1.62±0.08

[a] Density of 20 animals/plate for females, 25 animals/plate for males.

3.3. Analysis of age-specific mortality

Mean and maximum lifespans for *S. ratti* and for *C. elegans* hermaphrodites maintained under the same conditions and *C. elegans* hermaphrodites maintained in monoxenic conditions are given in Table 3. The mean lifespan of *C. elegans* in either culture condition was significantly longer than that of *S. ratti* (same conditions: $\chi^2=1074.0$, $P<0.0001$; monoxenic: $\chi^2=1201.0$, $P<0.0001$) (Fig. 3A). *C. elegans* mean lifespan was greater in monoxenic culture conditions than in *S. ratti* optimised culture conditions ($\chi^2=529.0$, $P<0.0001$), presumably due to the presence of contaminating microbes in the *S. ratti* culture conditions (Fig. 3A).

Limitation of lifespan by aging, rather than some other cause of mortality, may be established by testing for the occurrence of an exponential increase in mortality rate with increasing age (Wachter and Finch, 1997). In all these three experiments an age-specific mortality rate acceleration was observed. There was a significant positive linear relationship between ln ($m(t)$) and age for *S. ratti* and *C. elegans* under optimised culture conditions (*S. ratti*: $F_{1,14}=86.3$, $P<0.0001$; *C. elegans*: $F_{1,16}=45.7$, $P<0.0001$) and for *C. elegans* cultured monoxenically ($F_{1,23}=88.8$, $P<0.0001$ (Fig. 3B). The MRDT is significantly lower in *S. ratti* females than in *C. elegans* hermaphrodites, whether culture conditions in the latter were the same, or different to *S. ratti* ($t=3.3$, $P<0.01$; $t=3.8$, $P<0.001$, respectively) (Table 3). There was no difference in the MRDT for *C. elegans* under the two culture conditions ($t=2.0$, $P>0.05$). The IMR is greater in *S. ratti* females than in *C. elegans* hermaphrodites under either the same or different culture conditions ($t=2.0$, $P<0.001$; $t=13.8$, $P<0.001$, respectively) (Table 3). The IMR for *C. elegans* kept under *S. ratti* culture conditions is almost twice that of the monoxenically cultured populations ($t=5.0$, $P<0.001$) (Table 3). Overall, these results imply

Table 3
Mean and maximum lifespan, initial mortality rate (IMR) and mortality rate doubling time (MRDT) at 25 °C for virgin free-living female *S. ratti* and hermaphrodite *C. elegans* at optimum *S. ratti* conditions (1) and under monoxenic conditions (2)

Species	Condition	Lifespan ± SE (days)		IMR ± SE (10^{-3} days^{-1})	MRDT ± SE (days)
		Mean	Maximum		
S. ratti	1	3.0 ± 0.1	4.5 ± 0.8	25 ± 2	0.84 ± 0.09
C. elegans	1	7.7 ± 0.1	11.5 ± 2.1	3.6 ± 0.3	1.42 ± 0.15
C. elegans	2	11.2 ± 0.1	18.3 ± 4.3	1.8 ± 0.2	2.01 ± 0.30

that *S. ratti* is shorter lived than *C. elegans* because (a) it is more frail (as reflected by higher IMR), and (b) it ages more quickly (as reflected by a lower MRDT).

Using the mortality parameters in Table 3, it is possible to estimate what *S. ratti* lifespan might be, if it were possible to culture it monoxenically. For *C. elegans*, only the IMR differed significantly between the two culture conditions and was lower in monoxenic culture conditions. On monoxenic culture, *C. elegans* IMR is reduced by 50% relative to optimal *S. ratti* culture conditions. Substituting an *S. ratti* IMR reduced by a similar proportion into the equation ln ($m(t)$) against age, gives estimated median and maximum lifespans of monoxenically cultured *S. ratti* of 4.6 ± 0.8 and 5.3 ± 1.0 days, respectively. Alternatively, substituting the lower *C. elegans* IMR value into the equation ln ($m(t)$) against age, gives an upper estimate to median and maximum lifespans of monoxenically cultured *S. ratti* of 6.8 ± 1.5 and 7.7 ± 1.7 days, respectively. These contrast with observed median and maximum lifespans of 3.6 ± 0.6 and 4.5 ± 0.8 days, respectively.

3.4. Changes accompanying aging

First we examined age-related changes in feeding behaviour. In *C. elegans*, the rate of pharyngeal pumping declines gradually with age (Kenyon et al., 1993). In *S. ratti*, there was a reduction in both the rate of pharyngeal pumping ($F_{1,48} = 130.0$, $P < 0.0001$) and in the percentage of live animals pumping ($F_{1,2} = 62.3$, $P = 0.016$) (Fig. 4A) with age, consistent with typical nematode aging (Gems, 2002).

Second we examined age-related changes in movement. As with *C. elegans* (Herndon et al., 2002), *S. ratti* could be classified as class A (moving voluntarily), class B (only moving when prodded) or class C (not moving away but alive) behavioural phenotypes. There was an age-associated decline in movement (Fig. 4B). Specifically, on days 1 and 2 there were greater percentages of class A than class B worms (day 1: $\chi^2 = 11.4$, $P < 0.001$; day 2: $\chi^2 = 6.0$, $P = 0.014$) and greater percentages of class B than class C worms (day 1: $\chi^2 = 4.7$, $P = 0.03$; day 2: $\chi^2 = 5.3$, $P = 0.021$). On day 3 there were no differences in the percentages of the three behavioural classes (A vs. B: $\chi^2 = 2.6$, $P = 0.110$; B vs. C: $\chi^2 = 0.05$, $P = 0.827$; A vs. C: $\chi^2 = 3.0$, $P = 0.083$), but by day 4 classes B and C were predominating (A vs. B: $\chi^2 = 7.6$, $P = 0.006$; B vs. C: $\chi^2 = 3.3$, $P = 0.068$; A vs. C: $\chi^2 = 9.6$, $P = 0.002$) and by day 5 there were only class C worms remaining. Analysis of age-related changes in movement for 40 individual females found that of 32 class A animals, 26 proceeded through stages B and C before death, four became class C animals directly from class A and two died after becoming class B animals. Likewise, of 36 class B animals, 33 proceeded through stage C before dying.

We examined autofluorescence levels, which in *C. elegans* increase with age (Davis et al., 1982; Garigan et al., 2002). Microfluorometric measurements on one to

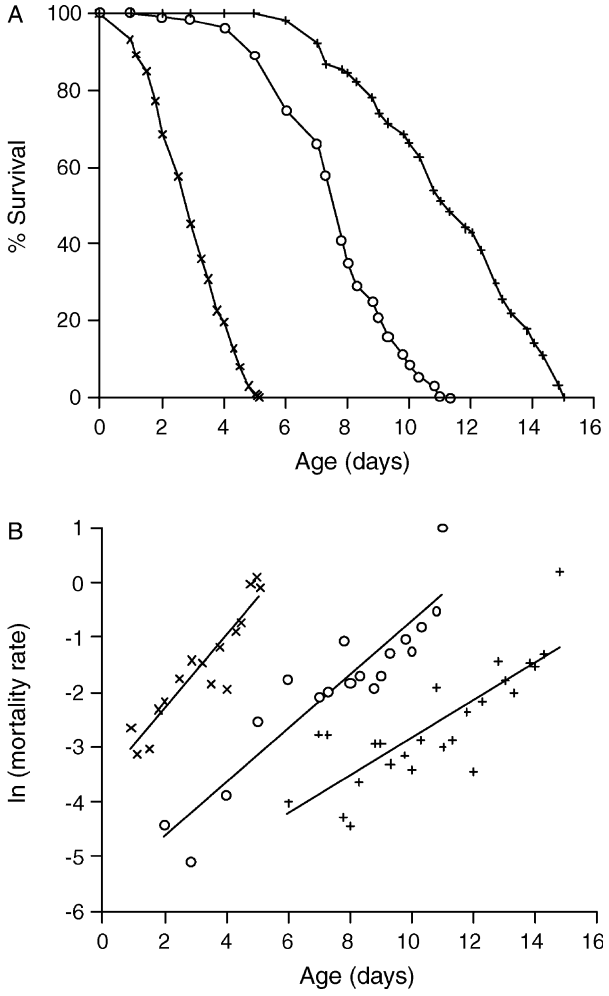
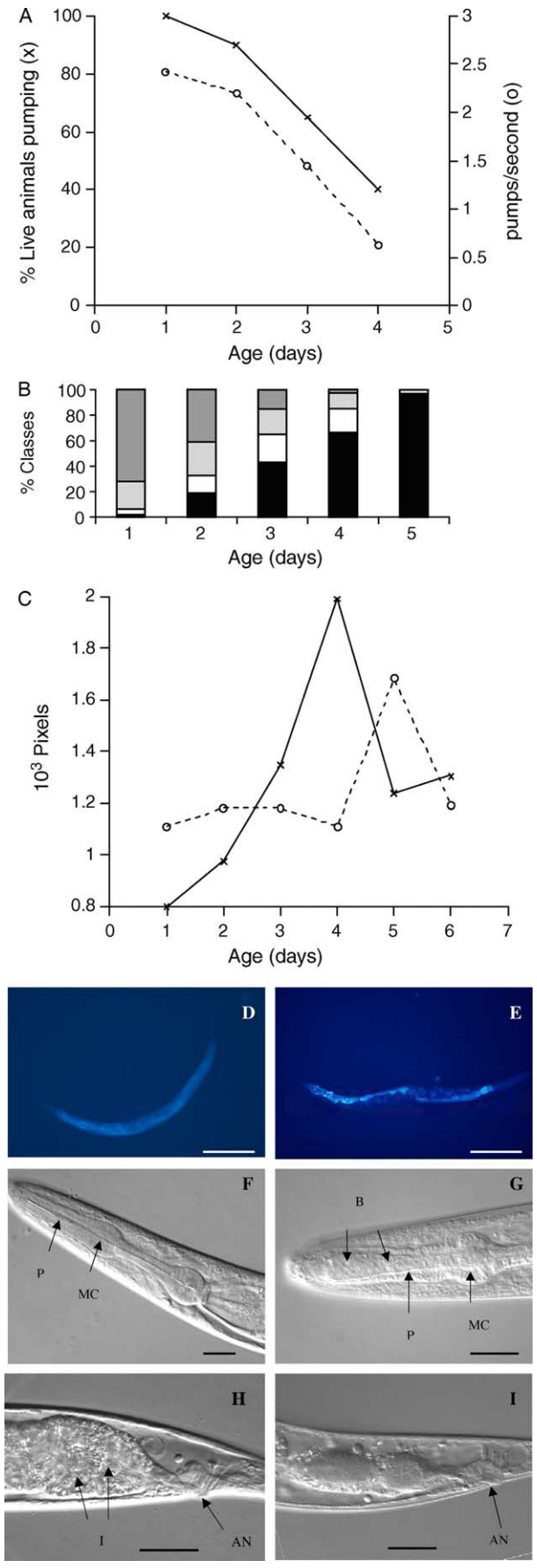


Fig. 3. (A) Survival and (B) plots of ln ($m(t)$) against age for *S. ratti* (x, —) and *C. elegans* (o, —) under optimised culture conditions and *C. elegans* (+, —) in monoxenic cultures.



six day old animals revealed significant increases in autofluorescence viewed using both DAPI ($F_{1,105}=18.2$, $P<0.0001$) (Fig. 4C–E) and GFP filters ($F_{1,109}=11.8$, $P<0.001$) (Fig. 4C).

Next we examined age changes in the appearance of the major nematode organs, comparing 1-day-old and 4-day-old adults observed with Nomarski microscopy (4-day-old adults, $N=8$). Overall, the appearance of aging *S. ratti* adults appeared similar to that of aging *C. elegans*, as previously described (Garigan et al., 2002; Herndon et al., 2002). In the main, the gross structural integrity of the major organs (e.g. the cuticle and reproductive system) was well preserved in older animals. However, in the pharynx, the majority of four day old animals showed swelling of the proporcus and metacorpus, which was often accompanied by the presence of large quantities of bacteria (Fig. 4G). Microbial blockage of the buccal cavity and pharynx has also been observed in aging *C. elegans* (Garigan et al., 2002). The most striking morphological changes occurred in the intestine. Whilst in 1-day-old animals there were large, healthy looking refractile intestinal cells (Fig. 4H), in the majority of four day old animals these cells were markedly atrophied and had a ragged appearance (Fig. 4I).

4. Discussion

In this study we document the phenomenology of aging in the very short-lived, free-living adults of the parasitic nematode *S. ratti*, comparing it with the longer-lived, well-characterised free-living nematode species *C. elegans*. One question that we wished to address was: is the short lifespan of *S. ratti* adults due to very rapid aging? Our results show clearly that it is.

This conclusion is supported by a number of observations. Most significantly, *S. ratti* exhibits an acceleration in age-specific mortality rate, one which is more rapid than that seen in *C. elegans* (Fig. 3B, Table 3). Several characteristics of organismal aging previously documented in *C. elegans* also occur in *S. ratti*, but at a faster rate. For example, with increasing age, levels of autofluorescence (lipofuscin) increase (Fig. 4C–E) and the rate of feeding

Fig. 4. (A) Mean pharyngeal pumping rate (pumps per second) (o, - - -) and percentage of live animals pumping (x, —) with age for female *S. ratti* at 25 °C. (B) Percentage of grouped worms of class A (dark grey), class B (light grey), class C (white) and dead worms (black) with age for female *S. ratti* at 25 °C. (C) Changes with age in autofluorescence viewed using DAPI (x, —) and GFP (o, - - -) filters in female *S. ratti* at 19 °C. (D–E) Increased autofluorescence during aging in *S. ratti* free-living females viewed with a DAPI filter; (D) one day old adult; (E) four day old adult. Bars = 200 μM. (F–I) *S. ratti* free-living adults viewed using differential interference contrast (Nomarski) microscopy. (F) and (H) one day old adults; (G) and (I), four-day-old adults. (F) and (G) Pharyngeal region. In (F), the entire pharynx is visible, in (G), the procorpus (P) and metacorpus (MC) of the pharynx are distended and packed with bacteria (B). (H) and (I): posterior intestine. In (H), note the large, healthy refractile intestinal cells (I). In (I), the intestine is strikingly atrophied. Anus (AN). Bars = 20 μM.

(Fig. 4A) and movement (Fig. 4B) decline. We observed an age-related decline in movement of *S. ratti*. In *C. elegans* a wide variation in the time of onset of reduction in movement was observed that supported the existence of substantial stochasticity in this trait. In *S. ratti* there was a less marked variation in time of onset of movement reduction, though about 20% of class A animals did die directly, consistent with some stochasticity. *S. ratti* lifespan and lifetime fertility are also affected by temperature (Fig. 2B and C) in a manner similar to that seen in *C. elegans* (Klass, 1977). Hence, the extreme brevity of life in free-living *S. ratti* adults, the shortest lived nematode thus far described, is the consequence of rapid aging, rather than some other, more swift and catastrophic life-shortening pathology.

Some aspects of aging in *S. ratti* differed from those in *C. elegans*. For example, in *S. ratti*, an increase in the ratio of males to females did not decrease female lifespan, whereas it does in *C. elegans* (Gems and Riddle, 1996), and in some other nematode species, e.g. *Ditylenchus triformis* (reviewed in Gems, 2001). In *C. elegans* this appears to be due to the effects of copulation rather than increased egg production (Gems and Riddle, 1996). In *S. ratti,* male lifespan was actually increased by exposure to females, whereas in *C. elegans*, mating greatly reduces male lifespan (Gems and Riddle, 2000). In the terrestrial nematode *Panagrellus redivivus*, mating shortens lifespan in females (as in *C. elegans*) but increased it in males (as in *S. ratti*). In *C. elegans*, grouping of males results in the formation of homosexual mating clumps, which approximately halves male lifespan compared with solitary males (Gems and Riddle, 2000). However, no such deleterious effects of male-male interactions were seen in *S. ratti* males, perhaps consistent with the absence of any observed clumping behaviour.

Why is the lifespan of this organism so short? In general, terrestrial nematodes such as *C. elegans* are very short lived, with maximum lifespans of the order of weeks to months. Among the longest lived is *Rhabditis tokai*, with a maximum lifespan of 145 days (Suzuki et al., 1978). By contrast, parasitic nematodes evolve much longer lifespans, with some hookworm and filarial nematode species attaining ages of more than a decade. Free-living *S. ratti* adult females are genetically identical to the adult parasitic females, yet the maximum lifespan of the latter is at least 11 months (Gemmill et al., 1997; Gardner, Gems and Viney, unpublished). Potentially, these evolved differences may reflect the differences in extrinsic mortality experienced by exposed free-living versus host-protected parasitic species. Yet it remains unclear why the lifespan of free-living *S. ratti* females is so much shorter that those of other terrestrial nematodes without parasitic morphs.

In conclusion, here we have documented age-changes in the free-living *S. ratti* morph; the shortest-lived nematode thus far described and one of the shortest-lived animal species, whose brevity of life appears to be the consequence of extremely rapid aging.

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References

Austad, S.N., 1993. Retarded senescence in an insular population of Virginia opossum (*Didelphis virginiana*). J. Zool. 229, 695–708.

Austad, S.N., Fischer, K.E., 1991. Mammalian aging, metabolism, and ecology: evidence from the bats and marsupials. J. Gerontol. A Biol. Sci. Med. Sci. 46, B47–B53.

Chapuisat, M., Keller, L., 2002. Division of labour influences the rate of ageing in weaver ant workers. Proc. R. Soc. Lond. Ser. B 269, 909–913.

Comfort, A., 1979. The Biology of Senescence, 3rd ed Elsevier, New York.

Cowan, S.T., Barrow, G.I., Feltham, R.K.A., Steel, K.J., 1993. Cowan and Steel's manual for the identification of medical bacteria. Cambridge University Press, Cambridge.

Davis, B.O., Anderson, G.L., Dusenbery, D.B., 1982. Total luminescence spectroscopy of fluorescence changes during aging in *Caenorhabditis elegans*. Biochemistry 21, 4089–4095.

Edney, E.B., Gill, R.W., 1968. Evolution of senescence and specific longevity. Nature 220, 281–282.

Finch, C.E., 1990. Longevity, Senescence and the Genome. University of Chicago Press, Chicago.

Garigan, D., Hsu, A.-L., Fraser, A.G., Kamath, R.S., Ahringer, J., Kenyon, C., 2002. Genetic analysis of tissue aging in *Caenorhabditis elegans*: a role for heat-shock factor and bacterial proliferation. Genetics 161, 1101–1112.

Garsin, D., Villanueva, J., Begun, J., Kim, D., Sifri, C., Calderwood, S.B., Ruvkun, G., Ausubel, F.M., 2003. Long-lived *C. elegans daf-2* mutants are resistant to bacterial pathogens. Science 300, 1921.

Gemmill, A.W., Viney, M.E., Read, A.F., 1997. Host immune status determines sexuality in a parasitic nematode. Evolution 51, 393–401.

Gems, D., 2001. Longevity and ageing in parasitic and free-living nematodes. Biogerontology 1, 289–307.

Gems, D., 2002. Ageing, in: Lee, L.D. (Ed.), The Biology of Nematodes. Taylor and Francis, London, pp. 413–455.

Gems, D., Riddle, D.L., 1996. Longevity in *Caenorhabditis elegans* reduced by mating but not gamete production. Nature 379, 723–725.

Gems, D., Riddle, D.L., 2000. Genetic, behavioural and environmental determinants of male longevity in *Caenorhabditis elegans*. Genetics 154, 1597–1610.

Gems, D., Sutton, A.J., Sundermeyer, M.L., Albert, P.S., King, K.V., Edgley, M.L., Larsen, P.L., Riddle, D.L., 1998. Two pleiotropic classes of *daf-2* mutation affect larval arrest, adult behavior, reproduction and longevity in *Caenorhabditis elegans*. Genetics 150, 129–155.

George, J., Bada, J., Zeh, J., Scott, L., Brown, S., O'Hara, T., Suydam, R., 1999. Age and growth estimates of bowhead whales (*Balaena mysticetus*) via aspartic racemization. Can. J. Zool. 77, 571–580.

Harvey, S.C., Gemill, A.W., Read, A.F., Viney, M.E., 2000. The control of morph development in the parasitic nematode *Strongyloides ratti*. Proc. R. Soc. Lond. Ser. B 267, 2057–2063.

Herndon, L.A., Schmeissner, P.J., Dudaronek, J.M., Brown, P.A., Listner, K.M., Sakano, Y., Paupard, M.C., Hall, D.H., Driscoll, M., 2002. Stochastic and genetic factors influence tissue-specific decline in ageing *C. elegans*. Nature 419, 808–814.

Hodgkin, J., Barnes, T.M., 1991. More is not better: brood size and population growth in a self-fertilizing nematode. Proc. R. Soc. Lond. Ser. B 246, 19–24.

Keane, J., Avery, L., 2003. Mechanosensory inputs influence *Caenorhabditis elegans* pharyngeal activity via ivermectin sensitivity genes. Genetics 164, 153–162.

Kenyon, C., Chang, J., Gensch, E., Rudener, A., Tabtiang, R.A., 1993. A *C. elegans* mutant that lives twice as long as wild type. Nature 366, 461–464.

Klass, M.R., 1977. Aging in the nematode *Caenorhabditis elegans*: major biological and environmental factors influencing life span. Mech. Ageing Dev. 6, 413–429.

Klass, M.R., Hirsh, D.I., 1976. Non-ageing development variant of *Caenorhabditis elegans*. Nature 260, 523–525.

Lakowski, B., Hekimi, S., 1998. The genetics of caloric restriction in *Caenorhabditis elegans*. Proc. Natl Acad. Sci. USA 95, 13091–13096.

Lyman, C.P., O'Brien, R.C., Greene, G.C., Papafrangos, E.D., 1981. Hibernational longevity in the Turkish hamster *Mesocricetus brandti*. Science 212, 668–670.

Martínez, D.E., 1998. Mortality patterns suggest lack of senescence in hydra. Exp. Gerontol. 33, 217–225.

McCay, C., Crowell, M., Maynard, L., 1935. The effect of retarded growth upon the length of the life span and upon the ultimate body size. J. Nutr. 10, 63–79.

Nusbaum, T., Rose, M., 1999. The effects of nutritional manipulation and laboratory selection on lifespan in *Drosophila melanogaster*. J. Gerontol. A Biol. Sci. Med. Sci. 54, B192–B198.

Page, R.E., Peng, C.Y.-S., 2001. Aging and development in social insects with emphasis on the honey bee, *Apis mellifera* L. Exp. Gerontol. 36, 695–711.

Roth, G.S., Lane, M.A., Ingram, D.K., Mattison, J.A., Elahi, D., Tobin, J.D., Muller, D., Metter, E.J., 2002. Biomarkers of caloric restriction may predict longevity in humans. Science 297, 811.

Schnabel, R., 1999. Microscopy, in: Hope, I.A. (Ed.), *C. elegans*, A Practical Aproach. Oxford University Press, Oxford, pp. 119–141.

Sherman, P., Jarvis, J., 2002. Extraordinary life spans of naked mole-rats (*Heterocephalus glaber*). J. Zool. 258, 307–311.

Sohlenius, B., 1969. Studies on the population development of *Mesodiplogaster biformis* (Nematoda, Rhabditida) in agar culture. Pedobiologia 9, 243–253.

Spieler, M., Schierenberg, E., 1995. On the development of the alternating free-living and parasitic generations of the nematode *Rhabdias bufonis*. Invertebr. Reprod. Dev. 28, 193–203.

Sulston, J., Hodgkin, J., 1988. Methods, in: Wood, A.B. (Ed.), The Nematode *Caenorhabditis elegans*. Cold Spring Harbor Press, New York, pp. 587–606.

Suzuki, K., Hyodo, M., Ishii, N., Moriya, Y., 1978. Properties of a strain of free-living nematode, *Rhabditidae* sp.: life cycle and age-related mortality. Exp. Gerontol. 13, 323–333.

Viney, M.E., 1994. A genetic analysis of reproduction in *Strongyloides ratti*. Parasitology 109, 511–515.

Viney, M.E., 1996. Developmental switching in the parasitic nematode *Strongyloides ratti*. Proc. R. Soc. Lond. Ser. B 263, 201–208.

Viney, M.E., Matthews, B.E., Walliker, D., 1993. Mating in the nematode parasite *Strongyloides ratti*: proof of genetic exchange. Proc. R. Soc. Lond. Ser. B 254, 213–219.

Wachter, K.W., Finch, C.E., 1997. Between Zeus and the Salmon. National Academy Press, Washington DC.

Wilkinson, G., South, J., 2002. Life history, ecology and longevity in bats. Aging Cell 1, 124–131.

Williams, G.C., 1957. Pleiotropy, natural selection and the evolution of senescence. Evolution 11, 398–411.

Yamada, M., Matsuda, S., Nakazawa, M., Arizona, N., 1991. Species-specific differences in heterogonic development of serially transferred free-living generations of *Strongyloides planiceps* and *Strongyloides stercoralis*. J. Parasitol. 77, 592–594.