AMMONIA, RESPIRATION, AND LONGEVITY IN NEMATODES: INSIGHTS ON METABOLIC REGULATION OF LIFE SPAN FROM TEMPORAL RESCALING

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ABSTRACT

To better understand metabolic correlates of longevity, we used a graphical technique to compare the adult temporal patterns of several markers of metabolic activity — ammonia elimination, oxygen consumption rate, ATP levels, and (in freeze-permeabilized worms) the rate of NADPH-activated, lucigenin-mediated superoxide formation — as observed by us and others in normal and long-lived mutant *Caenorhabditis elegans* strains. All of these traits declined with time, and when their logarithms were plotted against time, appeared reasonably linear for most of the duration of the experiments. The profiles for ammonia output conformed well to parallel regression lines; those for the other metabolic parameters varied widely in slope as originally plotted by the authors, but much less so when replotted as logarithms against *adjusted* time, scaled by the reciprocal of strain longevity. This is consistent with coregulation of life span, respiration rate, ATP levels, lucigenin reactivity, but not ammonia excretion, by a physiological clock distinguishable from chronologic time. Plots, scaled appropriately for equalized slopes, highlighted y-axis intercept differences among strains. On rescaled plots, these constitute deviations from the expectation based on 'strain-specific clock' differences alone. With one exception, y-intercept effects were observed only for mutants in an insulin-like signaling pathway.

INTRODUCTION

The soil nematode *Caenorhabditis elegans* is but one of several laboratory model systems providing new detail about metabolic aspects of organismal aging, with successes largely built upon prior discoveries of genetic variants with extended life (reviewed by Shmookler Reis & Ebert, 1996). The nematode model holds records for life extension (~5-fold), and for number of 'Age' genes, i.e., genes able to prolong life span when mutated or overexpressed (>3 dozen). Several *C. elegans* Age genes, and other genes with which they interact, have been cloned (Morris et al. 1996; Kimura et al. 1997; Ewbank et al. 1997; Ogg et al. 1997; Lin et al. 1997; Ogg & Ruvkun 1998; Paradis et al. 1999).

An Insulin-Like Signaling Pathway

Some of these genes had been found to interact, and thus, have been ordered along a bifurcated pathway (Vowels & Thomas 1992; Gottlieb & Ruvkun 1994; Dorman et al. 1995), quite similar to the signaling cascades activated by insulin and IGF-1 (insulin-like growth factor) in mammals (Tissenbaum & Ruvkun 1998; Paradis et al. 1999). The *daf-2* gene encodes an insulin/IGF-1 receptor family member (Kimura et al. 1997) that appears to phosphorylate both itself, and the regulatory subunit of a phosphoinositol 3-kinase, the catalytic subunit of which is encoded by the gene *age-1* (Morris et al. 1996). The pathway exerts its effects through *daf-16*, encoding a forkhead, or "winged helix", transcription factor family member (Ogg et al. 1997; Lin et al. 1997), that must be functional for longevity or any other phenotype of *daf-2* and *age-1* mutants to manifest (Kenyon et al. 1993; Gottlieb & Ruvkun 1994; Dorman et al. 1995; Larsen, et al. 1995; Gems et al. 1998). Other phenotypes include increased resistance to stress, notably, UV and thermal insult (Lithgow et al. 1995; Murakami & Johnson 1996); and constitutive formation of dauer larvae (*Daf-c*), the regulation of which can be said to be the primary function of the DAF pathway.

The Dauer Larva

Augmentation of adult longevity by *daf-2* and *age-1* mutations may well be an inadvertent side effect of DAF-pathway mutants, given the regulatory role of the DAF-2 pathway in dauer formation, and the population biologists' cogent case against developmentally programmed aging in iteroparous species (e.g. Rose 1991). Nematodes en route to reproductive adulthood traverse four larval stages (L1–L4), but the dauer larva is an alternative L3 stage, resulting from a developmental-arrest pathway initiated in L1 by high exogenous levels of a nematode pheromone, and proceeding via an L2d stage that preserves features of L1 and early L2 metabolism (reviewed by Riddle 1988, Riddle & Albert 1997). Dauer larvae can endure and/or escape harsh environments, including food scarcity, overcrowding, nematode waste buildup, dessication, hypertonicity, unfavorable pH, and chemical insult. All are tolerated, thanks both to a specialized cuticle (that also blocks the pharynx, anus, and 8 of 10 external sense organs), and to unique aspects of dauer metabolism.

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Dauer Metabolism

Unable to feed, dauer larvae rely upon β -oxidation of their abundant storage fat to yield both reducing equivalents for energy, and acetyl CoA (O'Riordan & Burnell 1989, 1990). The dauer cannot afford to waste carbon intermediates as CO_2 , so the eight reducing equivalents generated by a turn of the tricarboxylic acid cycle are forsaken, in favor of two, plus a succinic acid intermediate formed of two acetyl CoA molecules via the glyoxylate shunt (Wadsworth & Riddle, 1989; O'Riordan & Burnell 1989, 1990; Reape & Burnell 1990). In *Ascaris suum*, *Ancylostoma ceylanicum* and *Nippostrongylus brasiliensis*, glyoxylate shunt enzymes appear to be localized to mitochondria rather than peroxisomes or glyoxysomes (Rubin & Trelease 1976; Singh et al. 1992); this may also be true of *C. elegans*, since a survey of predicted *C. elegans* proteins gives mitochondria as the expected location for a putative isocitrate lyase, based on protein sequence motifs (Proteome online database).

Clk Genes

Four *C. elegans* genes are known, for which mutant alleles lead to dysregulation of the rates of development and behavior, but otherwise good health: *clk-1*, *clk-2*, *clk-3*, and *gro-1*. The *clk-1* gene has been cloned; it encodes a homolog of the yeast mitochondrial metabolic regulator Cat5p/Coq7p (Ewbanks et al. 1997), but its biochemical role is unknown (Hekimi & Lakowski 1999). Alleles of *clk-1* were shown by the Hekimi laboratory to exhibit slowed and more variable timing of embryonic and larval development, and also of adult processes such as food intake (pharyngeal pumping) and defecation (Wong et al. 1995; Lakowski & Hekimi 1996; Felkai et al. 1999). Total mean life span was also modestly extended for the *clk-1(qm30)* strain, by 23%, 20%, and 14% in the above three references respectively, and for *clk-1(e2519)*, by 40% and 7% in the first two papers. We performed a statistical resampling analysis on these survival data to isolate the effects on adult longevity (Thaden, unpublished), and found reduced effect and significance of the two *clk-1* alleles when their extended development times were subtracted from total life span. Neither allele was associated with adult life extension relative to N2 controls in our single determinations (Thaden et al. manuscript in preparation), while the *e2519* mutant has lived more than twice as long as N2, in axenic culture (Braeckman et al. 1999). We conclude that the effect of these *clk* mutations on adult life span is modest, and variable among experiments and laboratories, suggesting a strong interaction with environment.

Braeckman et al. (1999) also reported that the increased longevity of the *clk-1* strain was reversed in a *daf-16; clk-1(e2519)* double mutant. This has been the criterion by which other genes have been assigned to the DAF-2 signal transduction pathway, but such assignment may be premature in view of the disagreement among laboratories regarding the Age phenotype of *clk-1*.

Hekimi and colleagues have suggested that *Clk* genes perhaps constitute parts of a general physiological clock, mutations to which slow the pace of many or all life processes (Lakowski & Hekimi 1996; Hekimi et al. 1999) — which we take to mean that they proceed at a rate equal to chronological time multiplied by some constant less than one. This hypothesis is tested for the adult stage by our graphical analysis below.

Nitrogen Elimination

We surveyed a panel of Age strains and controls, measuring three parameters of *Caenorhabditis* nitrogen metabolism: elimination of nitrogen as ammonia and ammonium ion, elimination of amino acids, and incorporation of tritium during brief feeding on *Escherichia coli* previously cultured with ^3H amino acids (Thaden et al. in preparation). Ammonia-nitrogen and aminoacyl-nitrogen were produced in nanogram quantities per young adult, over a 3-hour assay, in roughly 2:1 ratio. They were also the major nitrogenous wastes found in the media of two other soil nematodes studied (Rothstein 1963; Wright 1975).

Figure 1 summarizes, for the purpose of graphical comparisons, some ammonia elimination results from a cohort of eight strains, three previously reported as long-lived, and five of normal longevity (Thaden et al. in preparation). All showed a greater-than-linear decline that could be fit with little scatter to parallel lines on a plot of the logarithm of ammonia output per unit of biomass as a function of adult age. They fell into two groups, depicted by the regression lines shown in Figure 1. The low group (dashed line) comprised all the strains with genotypes previously associated with longevity: *daf-2(e1370)*, *clk-2(qm37)*, and *daf-2(e1370) clk-1(qm30)*. The high group (solid line) averaged 2.1 times as much ammonia on adult day 5, for example, and included all tested strains of normal longevity: wild types Bristol N2,

Figure 1. Regression summary of the adult temporal patterns of ammonia-nitrogen elimination (based on data from Thaden et al., manuscript in preparation), for groups of five strains with normal life span (solid line and filled circles) and three strains containing mutations that enhance longevity (dashed line, open circles).

Bergerac BO, Cl2a, and RC301; and the *daf-16(m26); daf-2(e1370)* double mutant. The apparently slightly steeper slope for the longevity genotypes was not significant ($P=0.093$).

Ammonia Metabolism

Biological roles of ammonia and ammonium ion have been reviewed (Wright 1995). Ammonia is produced by animals primarily through catabolism of dietary protein. Unlike lipids and sugars, excess amino acids are not stored, but are transaminated to glutamate, which is oxidatively deaminated to yield NH_4^+ . Deamination is catalyzed by glutamate dehydrogenase, also a major allosteric regulator affecting ammoniogenesis.

Ammonia is used to modulate pH by organisms; in the event of acidosis, it is synthesized and excreted by the mammalian kidney. The pK of the $\text{NH}_3/\text{NH}_4^+$ acid-base pair is 9.5. NH_3 easily permeates most biological membranes, so ammonia elimination by nematodes and other small animals may be by passive diffusion. NH_4^+ does not passively cross lipid bilayers, but is readily shunted by transporters of potassium ion, notably Na^+ , K^+ -ATPases. Differential membrane permeability to NH_3 and NH_4^+ can cause "diffusion trapping" in the presence of a pH gradient: protonation of NH_3 in the acidic compartment leads to ammonia accumulation. Diffusion trapping may set an upper limit to environmental pH for ammonotelic animals.

Ammonia Toxicity

Ammonia is the major excretion product of most aquatic, but few terrestrial, animals because each gram of ammonia-N (nitrogen in NH_3 or NH_4^+) must be diluted by ~500 ml of water to render it nontoxic (Wright, 1995). In mammals, where ammonia is transported as glutamine in the blood, hyperammonemia associated with liver failure or familial ornithine transcarbamylase (OTC) deficiency can produce coma and death. In rats, ammonium acetate injection produced a 5-fold increase in brain mitochondrial ammonium ion, and led to serious disturbances in the electron-transport chain and the malate-aspartate shuttle: notably, severe ATP depletion, and decreased State 3 respiration rates and respiratory control in isolated brain mitochondria (Kosenko et al. 1996, 1999). The picture appears to be somewhat different for rat astrocytes, where ammonia-associated swelling and decreased energy metabolism may occur by a less direct route: ammonia stimulation of glutamine levels leading to osmotic effects (Dolinska et al. 1996; Willard-Mack et al. 1996).

Respiratory Metabolism as a Life-History Trait: Effects of Longevity-Confering Mutations

The ammonia elimination result summarized in Figure 1 contrasts in an interesting way with temporal profiles of other metabolic parameters in the literature. We chose Braeckman et al. (1999) because this publication of the Vanfleteren group, primarily focused on Clk mutants, contains a striking and important, although indirect, revelation about the Daf-associated Age phenotype, evident

in their plots but better appreciated upon graphical re-analysis of their data, a portion of which is included here. This publication has also been reviewed by Gems (1999).

Braeckman et al. (1999) describe three experiments. Cultures of 3–5 strains were begun simultaneously from eggs and switched as pre-adults to liquid culture. Samples were taken daily, cleared of dead worms, and used to assay three metabolic parameters: respiration rate as measured by oxygen consumption; steady-state ATP levels; and "metabolic potential", defined as luminescence induced by oxidation of lucigenin, apparently by superoxide, in permeabilized worm suspensions also containing NADPH. Results were normalized to total protein, and displayed along logarithmic y-axes, against adult age.

For the single Clk mutants *clk-1(e2519)* and *gro-1(e2400)*, oxygen consumption was reported as mildly reduced relative to N2 wild type, during the first 4–5 days of adulthood but not thereafter. Aged Clk mutants eventually retained higher metabolic capacities than N2. The authors observed more distinctly elevated ATP levels in aging *clk-1* and *gro-1* strains relative to N2, an effect markedly accentuated in the presence of either the *daf-2(e1370)* or *age-1(hx546)* canonical allele within double mutants. Thus, the conclusion of their title: energy production and consumption appear to be uncoupled in Clk mutants. Finally, the authors note that *daf-16; clk-1* double mutants have normal life span and ATP levels, but reduced respiration rate.

Slope differences dominate the figures of Braeckman et al. (1999), and are the changes chiefly addressed by the authors. Because longer-lived strains consistently displayed shallower declines with time, they suggested that physiological time is retarded relative to chronological time in Clk mutants, in accord with the slowed development and behavior of these strains (Wong et al. 1995; Lakowski & Hekimi 1996).

Slope-Normalization by Temporal Rescaling

The slope differences intrigued us, particularly when viewed from the perspective of our own data. As noted above, we had observed striking log-linear declines in ammonia elimination as a function of *C. elegans* adult age, but not genotype, indicating nearly identical slopes among eight strains varying in life span over a 2.5-fold range. One consequence for us was that, on our plots, another aspect of the data stood out: two- to three-fold, *age-independent* differences in ammonia elimination among the strains — that is, slope-preserving, vertical shifts in the age profiles (and hence, in the y-axis intercept), that themselves were associated with life span (summarized in Figure 1, derived from data of Thaden et al. in preparation).

Temporal patterns of both types, scaled to life span and not, have been reported in a series of papers by Helfand and colleagues for *Drosophila* gene expression, including an early one cited by Braeckman et al. for its method of graphical display with the x-axis expressed as percentage of the maximum life span lived (Helfand et al.

Figure 2. Graphical reanalysis of results of Braeckman et al. (1999, Figures 1a, 2a, and 3a), with all units of time normalized to the Bristol wild-type strain (N2), i.e., with time units replaced by the quotient of time divided by the relative life span (rLS) of each strain (relative to N2), as listed in panel (a). Plot ordinates are the logarithms of (a) respiration rate, expressed per N2-normalized hour (h*); (b) superoxide production rate, as measured by NADPH-activated oxidation and subsequent luminescence of lucigenin by freeze-permeabilized worms, also expressed per N2-normalized hour (h*); and (c) steady-state ATP level. These parameters are all plotted against 'N2-equivalent days' of adulthood, i.e., the quotient of actual days divided by a strain's rLS. For calculation of rLS values listed in panel (a), survival data were unavailable under comparable (mass monoxenic culture) conditions, but, with one exception (†), were at least available from the same report (Braeckman et al. 1999, Table 1), albeit with worms in small axenic cultures. For the exception, the rLS value (†) was derived from a survival assay conducted upon nematodes maintained on bacterial lawns on agar (Thaden et al. manuscript in preparation).

1995). This presentation was not utilized in the Braeckman et al. (1999) study for a technical reason.

In Figure 2, we have replotted data extracted from the left panels of each of Figures 1, 2, and 3 of Braeckman et al. (1999), with three modifications: (i) the means of their triplicate observations are treated as single observations (the full data could not be extracted from their plots); (ii) the time axis is scaled by the reciprocal of the life span of each strain relative to that of the N2 wild-type control (estimated as described below), thus effecting a

conversion to units of 'N2-equivalent days'; and (iii) the two metabolic parameters that are expressed 'per hour' (Figure 2, y-axes of Panels a and b) were converted to rates 'per N2-equivalent hour', again using reciprocal relative life span. Since life spans in monoxenic suspension mass cultures had been neither measured in the actual experiment, nor published elsewhere for these strains, we had to approximate relative life spans (listed in Figure 2a) using survival data gathered under other culture conditions: for the *clk-1* and *daf-16; clk-1* strains, mean life span in small axenic liquid cultures as reported by the authors (Braeckman et al. 1999, Table 1); and for the *daf-2 clk-1* strain (not in their Table 1), median life span in monoxenic cultures on agar (Thaden et al. in preparation). Both included simultaneous N2 controls. Since relative life span (relative to a simultaneous control) is likely to be more robust in the face of culture variables than absolute life span itself, we scaled to 'N2-equivalent' time, rather than 'percentage of life span'.

As anticipated by Braeckman et al. (1999), life-span scaling (Figure 2) produces steeper profiles for Age strains *clk-1* and *daf-2 clk-1*, relative to N2, than does the chronological scaling in their original figures. Most profiles appear fairly linear, as on their original plots, through N2-equivalent day 6 or 7 (or slightly convex, for ATP levels in Figure 2c, suggesting a decline that is a bit less than logarithmic), but now with comparable steepness among the strains in each frame. This suggests that the reciprocal of strain life span, even crudely determined from unrelated experiments, correlates well with the strain differences in steepness of the age-dependent decline for these parameters.

After N2-equivalent day 7, the profiles flatten out, or show transient spikes (*clk-1*). These late features may reflect a real, discrete change in the rate of metabolic decline in post-fertile adults; or simply population selection for more vigorous individuals during age-associated mortality (Van Voorhies & Ward 1999); or presence of some inadvertent (breakthrough) progeny that would contribute disproportionately to assay results (Braeckman et al. 1999); or day-to-day assay variation exacerbated by low signal-to-noise ratios in these later assays. We interpret the coincidence of these changes for different strains on our rescaled plots as further evidence that the temporal profiles of these three metabolic metrics are sensitive to strain longevity.

Excluding observations beyond N2-equivalent day 7, an analysis of variance was used for each Panel of Figure 2 to test the null hypothesis of equal slopes among strains. (Because the number of observations per strain ranged from $n = 8$ to $n = 15$, each observation was weighted by $1/n$ while fitting the consensus regression slope, which therefore appropriately represents the four strains.) A Bonferroni correction was applied, to allow for the 3 comparisons made, giving $\alpha = 0.017$ for a family-wise confidence level of 95%. By this criterion, the equal-slopes hypothesis is not rejected for respiration rate (Figure 2a, $P = 0.026$), nor for metabolic potential (Figure 2b, $P = 0.109$), nor for ATP level

(Figure 2c, $P = 0.055$). Equal slopes are consistent with the authors' suggestion that these metabolic parameters are controlled by a physiological clock operating at a fractional rate of chronological time — also the explication of *Clk* phenotypes favored by Hekimi and colleagues (e.g. Lakowski & Hekimi 1996) — and with strain-specific rates for this physiological clock proportional to the reciprocal of our admittedly rough estimates of strain relative life span. The best-fit consensus logarithmic slopes for the three parameters are equivalent to declines *per N2-equivalent day* of 20% for oxygen consumption (Figure 2a, $m = -0.09737$, $R^2 = 0.912$), 42% for light production (Figure 2b, $m = -0.2371$, $R^2 = 0.929$), and 16% for ATP levels (Figure 2c, $m = -0.7510$, $R^2 = 0.884$).

The real value of a scaled analysis is its potential to emphasize features otherwise obscured by massive slope differences. In Figure 2a, the most striking difference is the uniformly higher values for the *daf-2 clk-1* strain relative to N2 and the other strains. The *daf-16; clk-1* strain, on the other hand, is somewhat lower than N2 after the first two determinations. Although starting and ending high, the *clk-1* strain otherwise loosely tracks with N2. Figure 2b shows tight correspondence among three strains over most of the experiment, with the disparate strain again being *daf-2 clk-1*, but now with lower values over much of the experiment. Note that luminescence spans the widest range of values, so the visually small deviation of *daf-2 clk-1* is not trivial. In Figure 2c also, the distinctive temporal pattern is that of *daf-2 clk-1*, with ATP levels elevated by a constant factor across its life span. A synthesis emerges from these three metrics of metabolism performed on the same cohort. The effect of the *clk-1(e2519)* mutation upon respiration, metabolic potential, and ATP levels is entirely or predominantly one of profile-stretching, consistent with general slowing of life processes. However, *daf-2(e1370)*, at least in the context of *clk-1*, acts to uniformly raise respiration rate and ATP levels, and uniformly lower the potential to elicit luminescence from lucigenin, throughout adult life. This implicates the *Daf* signaling pathway as a regulator also of respiratory metabolism. Consistent with this view is the observation that oxygen consumption is lowered in the *daf-16; clk-1* strain, since *daf-16* mutations would presumably also abrogate the wild-type *Daf-2* effects in feeding worms. The role of the *Daf* pathway as a regulator of respiratory metabolism was also highlighted by Vanfleteren et al. (1998).

It is difficult to compare the vertical shifts on one panel of Figure 2 to those on another because of the different log scales, so in Table 1, we have re-expressed these vertical shifts as fractions of the N2 value at the same relative point in their adult life, i.e. at the same physiological age — this applies to *any* physiological age, under the equal-slopes hypothesis — and have included the results of pairwise statistical tests of these vertical shifts, corrected for multiple comparisons. Note that these statistical test results are only as valid as our estimates of relative life span used for data replotting, estimates

Table 1.

	Respiration Rate	Lucigenin Oxidation	ATP Level
N2	1	1	1
<i>clk-1</i>	1.25 (0.91-1.73)	0.85 (0.62-1.17)	0.94 (0.68-1.29)
<i>daf-16; clk-1</i>	0.77 (0.55-1.04) [†]	0.74 (0.54-1.02)	0.90 (0.66-1.24)
<i>daf-2 clk-1</i>	1.62 (1.17-2.24) [†]	0.53 (0.38-0.73) ^{†,‡}	2.03 (1.47-2.81) ^{†,‡}

Values shown are fractions of the N2 value, with 95% confidence limits.

[†]Significantly different from N2 ($P < 0.0005$). [‡]Significantly different from the *clk-1* strain ($P < 0.0005$). Absence of these symbols means the value did not differ significantly from those controls ($P > 0.05$). The probabilities and confidence bounds shown are corrected by the Bonferroni method for the 15 comparisons (of strains to N2 and to *clk-1* controls).

necessarily derived from survival determinations in other culture conditions.

The *clk-1* strain showed no significant deficits of respiration rate, lucigenin luminescence, or ATP level in a scaled comparison with N2, meaning these values are those expected with simple slowing of a physiological clock. The *daf-16; clk-1* double mutant likewise did not differ significantly from N2 in any of the three parameters, but was significantly lower than *clk-1* in respiration rate.

In the *daf-2 clk-1* double mutant, ATP levels were increased more than 2-fold over N2 and *clk-1* (each $P < 0.0005$). The oxygen consumption rate of *daf-2 clk-1* worms was increased 1.6-fold over N2 ($P < 0.0005$), but its increase over *clk-1* was not significant ($P = 0.3$). Because oxygen consumption and ATP levels both rose a similar amount in *daf-2 clk-1*, and not at all in *clk-1*, one needn't invoke uncoupling of the production and utilization of energy in *Clk* strains, as argued by Braeckman et al. (1999). Most interestingly, however, the potential to generate lucigenin luminescence moved in the opposite direction for the *daf-2 clk-1* strain, falling to half that of the N2 strain, i.e., half the value predicted by a simple slowed-clock hypothesis. In a previous report, long-lived *age-1* and *daf-2* single mutants showed higher lucigenin light production than N2 at all ages, starkly contrasting with the effect of *daf-2* in the context of *clk-1* (our Figure 2b), and also displayed higher activities of both the TCA cycle enzyme isocitrate dehydrogenase, and the glyoxylate shunt enzyme isocitrate lyase (Vanfleteren & De Vreese 1995). It would be interesting to see these earlier data displayed on plots scaled to reciprocal life span.

Although *daf-2(e1370)* forces dauer formation upon a larva raised at restrictive temperature, the high steady-state ATP level in the *daf-2 clk-1* adult raised at permissive temperature is decidedly not a dauer-like feature. Wadsworth and Riddle (1989) measured soluble phosphorus species in worm perchloric acid extracts, by ³¹P NMR. The ATP fraction was below their level of detection (not stated) in extracts of either N2 *wt* or *daf-2* dauer larvae, but represented 7%, 7%, and 10% of soluble phosphorus in N2 larvae at the L2, L3 and L4 stages. As with dauers, ATP was undetectable in N2 L1 larvae, and in L2d pre-dauers of the *daf-2* mutant strain. Adults were not included in their study. In the N2 and *daf-2* dauers, inorganic phosphate was very high (90% and 59% of

total soluble phosphorus, respectively). From this and measurements of glyoxylate and TCA cycle key enzyme activities (Khan & McFadden 1980, 1982; Wadsworth & Riddle 1989), Wadsworth and Riddle concluded that L2d pre-dauers and dauers do not deploy the TCA cycle, as do L2 and L3 larvae.

An original intent of Braeckman et al. (1999) was to investigate epistatic interactions of these mutations. With data displayed this way, we show that this is indeed possible, but the most interesting epistases remain to be investigated: those that may impact upon the striking increase in steady-state ATP levels, and significant decrease in lucigenin oxidation potential, in the face of essentially unchanged respiration rate, associated with presence of either *daf-2(e1370)* (Table 1), or *age-1(hx546)* (reanalysis not shown). The authors are of course in a better position to continue in this vein.

DISCUSSION

New Insights into Age Phenotypes

Implications of a two-to-three-fold reduction in ammonia-nitrogen elimination by some Age strains but not others will be discussed in detail elsewhere (Thaden et al. in preparation). Our view is that nitrogen excretion (as opposed to reutilization) may be one of a number of nonconservative processes that, in the face of food removal such as occurred in our 3-hour assay, can be turned off more rapidly or effectively in dauer-constitutive mutant strains.

The *daf-2 clk-1* double mutant, but not the *clk-1* mutant, was 1.6- to 2-fold higher in respiration rate and ATP levels than N2, both normalized by life span. We suggest that these results for respiration rate differ from that reported by Braeckman et al. (1999) primarily because life-span scaling also affects the vertical displacement (y intercept) of any parameter that is expressed as a rate. Our reanalysis indicates that uncoupling of the production and utilization of energy may not be supported by the data. However, our values (Table 1) introduce a new paradox: The lucigenin reactivity is also a rate, and hence was also multiplied by the *daf-2 clk-1* life-span factor of 2.8, yet in spite of this, light production dropped 1.9-fold below the simple prediction of a slowed clock for *daf-2 clk-1* worms. This assay measures superoxide production potential (Vanfleteren and De Vreese 1996). Mitochondria can produce an oxyradical flood out of proportion with respiration and ATP synthesis if the ability of the inner membrane to maintain an electrical potential is compromised, for instance, by presence of ionophore reagents in the lipid bilayer (Nohl et al. 1997). Since the lucigenin assay is performed upon freeze-permeabilized worms, it is questionable to what extent mitochondrial membranes retain integrity, but, considering that *daf-2* strains are thermotolerant and generally stress-tolerant (Lithgow et al. 1995, Murakami & Johnson 1996), it may be that mitochondria of the *daf-2(e1370) clk-1(e2519)* mutant survive freezing better than those of the *clk-1* and other strains, and thus some of the

potential generators of superoxide either remain shielded from the lucigenin/NADPH solution, or remain properly coupled to ATP production and low oxyradical leakage. Alternatively, reduced potential to generate lucigenin luminescence by the *daf-2 clk-1* strain may be due to higher levels of superoxide dismutase (Vanfleteren & De Vreese 1995), specifically MnSOD, since the assay mixture contained 5 mM KCN (Braeckman et al. 1999), that might effectively compete with lucigenin for available superoxide radical during the assay, in spite of control experiments that argue otherwise (Vanfleteren & De Vreese 1996). The contradictory association of *higher* lucigenin light production with a *daf-2* single mutant in a prior experiment (Vanfleteren & De Vreese 1995) implies that there may be epistatic interaction between *daf-2* and *clk-1*, but these single and double mutants have yet to be compared simultaneously in a single light-production experiment.

We reviewed the dauer state because adult *daf-2* and *age-1* animals are in several visible respects dauer-like, and we suggest that this extends also to partial retention of the conservative metabolism of L1 and dauer larvae. For instance, a relatively large role of the glyoxylate cycle in *daf-2* reproductive adults may be one reason for an apparent paradox — 1.6-fold higher oxygen consumption (Figure 2 and Table 1), but ~2-fold lower carbon dioxide production (Van Voorhies & Ward 1999), by *daf-2(e1370) clk-1(e2519)* compared to Bristol N2 — since the glyoxylate shunt avoids CO₂ production. We suggest a second reason for this inconsistency: CO₂ output, like O₂ consumption, was expressed as a rate per hour. CO₂ output normalized to “N2-equivalent” hours is expected to be ~2.8-fold higher. Finally, CO₂ production was expressed per worm, and thus should be normalized to units of biomass before making direct comparison with O₂ consumption expressed per mg of protein; if *daf-2 clk-1(e2519)* is a smaller worm than N2, then CO₂ production will have been reported as low. In our hands, *daf-2 clk-1(e2519)* worms had 80% (± 10%, n = 3) as much total protein as N2 on the first day of adulthood (Thaden et al., in preparation), so this discrepancy in units may only modestly contribute to an explanation of the inconsistency in respiratory metabolism estimates by CO₂ vs. O₂ measurements in different laboratories.

Replotting

This paper demonstrates the utility of displaying temporal patterns of traits on axes of both chronological time and time scaled to life span, if the traits have been measured in test groups of differing longevity. The concept of appropriately scaling time axes to align and compare temporal profiles is not novel (e.g. Shock et al. 1984), and the scaling factor needn't be maximal life span as used by Helfand et al. (1995), nor relative mean and median life span, as we use here, nor even life span at all. One might use decline in frequency of locomotor body waves, for instance, quite possibly a superior gauge of *C. elegans* intrinsic aging rate than life span

(Bolanowski et al., 1981). We show that even ad hoc estimates of life span, from unrelated experiments, can “sweep out” the large effect of variable senescence among test groups so that other features of the data are apparent, notably, time-independent strain differences which themselves might correlate with life span. Without replotting, there is a risk of reporting again and again, in one trait then the next, the basic fact of an altered intrinsic pace to senescence.

Besides our preference for normalization by *relative average*, instead of *absolute maximal* life spans, our replotting method differs from Helfand's et al. (1995) in a second important feature: we apply temporal rescaling also to the time unit of any y-axis parameter that is measured as a *rate* (in the present study, respiration rate and superoxide generation rate, Figure 2, panels a and b). This is reasonable both mathematically and scientifically if one considers the limited objective of rescaling, that is, to test the hypothesis that something like a physiological clock, paced differently for different strains and approximated by the reciprocal of relative life span measurements, regulates the parameter under study. If this appears to be true, as adjudged by conformity of rescaled temporal patterns to parallel regression lines or curves (see Figure 2), then it is reasonable to conjecture that the physiological clock also acts upon the parameter of interest over the short term, as during an assay for the parameter. In other words, a second, expected result of a slower physiological clock upon an affected metabolic rate is to depress it, relative to strains with faster clocks, compared at the same point in their life spans; and conversely, a metabolic rate that is equal, for instance at adult day zero, but which subsequently declines more slowly for the longer-lived strains, constitutes an unexpected result. Note that the y axes in the rescaled plots of Figure 2a and b are no longer rates at all, but rather, the product of a rate and a temporal scaling factor, rLS; differences observed along these axes are not rate differences, but deviations from the expectations of a physiological clock hypothesis.

Implications of Log-Linear Regressions

All four metabolic parameters we discuss declined drastically with adult age, in log-linear fashion, defining a constant fractional loss per unit of time. This pattern is expected for reactions in a closed system, but why for metabolic processes, in the face of a replenished (or vast-excess) supply of food? Ultimately, it may reflect an evolutionary strategy for energy deployment which has been shaped by the predominant effect of early reproduction on exponential rates of population growth. The log-linear decline implies that some reserve is being depleted at a rate dependent on the amount remaining, but tells us nothing concerning the contribution of this reserve, positive or negative, to survival.

The steepness of the linear slope, indicating the fractional change per unit time, varies >15-fold among the four parameters (notice the vastly different y-axis scales), but insignificantly between long-lived and con-

trol strains for any one parameter, *provided* that the time is appropriately selected as either chronological (for ammonia-N production) or physiological — i.e., scaled to longevity (for ATP levels, oxygen consumption, and lucigenin oxidation). Any model which attempts to explain the pleiotropic effects of Age mutants will need to account for the vastly different rates of decline of metabolic parameters, while reconciling the fact that three such parameters all seem to be regulated by an intrinsic aging clock. Although such a model is beyond the scope of this review, a useful analogy for the large observed rate differences (apparent in differing slopes) would be to consider two chemical reactions in a closed system, dependent upon the same limiting reactant, but one utilizing it once, and the other five times, in its stoichiometry; reaction rates would thus depend on the concentration of this component to the first vs. fifth power.

The metrics of metabolism we discuss fall into two temporal pattern classes, identical to those noted previously by Rogina & Helfand (1996) for enhancer-trap lines of *Drosophila*: those for which temporal changes scale with life span and therefore seem to be regulated, however indirectly, by an intrinsic aging rate, and those for which temporal patterns change with age, but are unaltered in the face of life extension. Regarding the epistatic relation among these types of parameters, we are unable to make a strong inference, only a numerical one: since any metric which depends on a physiologically-scaled variable will in turn also reflect physiological age, those markers which cease to be predictive of longevity when scaled to physiological age are *likely* to be secondary to some aspect of physiological decline. The same cannot be said of markers such as ammonia production, which mirror absolute time, but this is not an argument for their primacy in the etiology of senescence.

An important property of these log-linear regressions is that they track in parallel for each parameter, although displaced vertically for certain genetically long-lived strains (for *daf-2 clk-1(e2519)* in the case of all three parameters of oxidative metabolism, for *daf-16*; *clk-1* oxygen consumption rate alone, and for the *daf-2*, the *clk-2*, the *daf-2 clk-1(qm30)*, and the *daf-2*; *daf-12* strains with regard to ammonia excretion), when plotted “appropriately” against either physiological or chronological time. These parallel shifts allow us to easily compare the total lifetime integrals among strains, because they reflect a constant ratio throughout much or all of the “adjusted” life span, such that the longest-lived *daf-2* strains *always* excrete less ammonia and consume more oxygen, etc., than control strains. Due to the steepness of the observed log-linear declines, late-life time points contribute rather little to the integrals under lines extrapolated to mean or maximal longevity, and hence can usually be ignored; as a consequence, such integrals are relatively insensitive to even quite large differences in longevity. Thus, *lifetime total* energy expenditure, oxygen consumption, and nitrogen production, all principally reflect levels obtaining in the first week of life, and are altered in long-lived mutants to

almost the same extent as the y-intercepts are displaced. Such changes are difficult to reconcile with simple rate-of-living theory. For example, if longevity were to hinge on lifetime metabolic expenditure as reflected in any of the metabolic parameters observed to undergo sharp log-linear declines, then the effect on longevity of a temperature shift (or of caloric restriction) should be greatest early in life, and may dwindle toward zero as it is implemented later and later. Thus far, most temperature-shift experiments in *C. elegans* (Hosono et al. 1982; Larsen et al. 1995) or *Drosophila* (Maynard Smith 1963; Miquel et al. 1976; Lints 1989), and delayed-onset caloric restriction studies in mice (Goodrick et al. 1990; Weindruch et al. 1996), are at odds with this contention.

Metabolic Stress: It Isn't All Oxidative

Oxidative damage is not the only stress imposed by metabolic activity. ATP-exhaustion can lead to deficiencies in vital repair processes, including DNA repair, protein renaturation by chaperonins, and pH and ion-balance perturbations. We reviewed evidence that ammonia is an energy poison in mammalian brain, where anabolic demands are high, leading to coma and death. The organelle affected is the mitochondrion and the result is ATP depletion and hypertonic swelling. Whether nematodes are susceptible is not known, but a common feature of their natural life is extreme crowding when a food bonanza nears exhaustion, with consequent rise in environmental ammonia and spikes in pH. We suggest it is the pH that is the primary problem, since the worm, now the acidic compartment, will concentrate ammonia by diffusion trapping. The large extensions of life span observed in nematode mutants may not be applicable to mammals, which are ureotelic rather than ammonotelic, and which already outlast even the longest-lived nematodes by several orders of magnitude. The shortest life spans, and the greatest potential for extension thereof, might plausibly be expected in ammonotelic organisms. The dauer pathway itself, which is strongly elicited in response to crowding, may have evolved in response to ammonia toxicity, which it largely prevents or ameliorates.

Generalized stress-resistance of Age mutant worms (e.g., *age-1* and *daf-2*; Lithgow et al. 1995; Murakami & Johnson 1996) may derive directly from their markedly decreased levels of toxic nitrogenous end-products and superoxide generation, thereby reducing the total load on stressed worms (see Sohal et al. 2000). It is thus an inherent property of the Age mutant phenotype of reduced metabolic productivity, that they should be both long lived and stress resistant. These may simply be two sides of the same coin.

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