Apparent uncoupling of energy production and consumption in long-lived Clk mutants of *Caenorhabditis elegans*

Bart P. Braeckman, Koen Houthoofd, Annemie De Vreese and Jacques R. Vanfleteren

Clk mutants of *Caenorhabditis elegans* are characterised by an overall slow down of temporal processes and increase in life span. It was hypothesised that Clk mutations slow down the pace of many cellular functions and lower the rate of energy metabolism, possibly resulting in slower production of reactive oxygen species which in turn could result in slower ageing [1–3]. We tested this hypothesis by measuring respiration rates, light production capacities [4] (a measure of metabolic potential) and ATP levels in various strains harbouring mutant alleles of the Clk genes *clk-1* and *gro-1* and of three other genes that interact with the Clk genes. We found a mild reduction of oxygen consumption rates but little alteration of metabolic capacities in the single Clk mutants during the first 4–5 days of their adult lives, relative to the wild-type strain. This difference tended to fade away with increasing age, however, and aged Clk mutants eventually retained higher metabolic capacities than the wild-type control strain N2. These profiles are suggestive of physiological time being retarded, relative to chronological time in Clk mutants. Ageing *clk-1* and *gro-1* mutants also retained substantially elevated ATP levels relative to the N2 strain, and the simultaneous presence of mutations in *daf-2* or *age-1* – genes that affect longevity – boosted this effect. Thus, energy production and consumption appear to be uncoupled in these mutants. Mutation in the transcription factor *daf-16* suppressed the Age and ATP phenotypes, but not the reduction of respiration rate imparted by mutation in *clk-1*.

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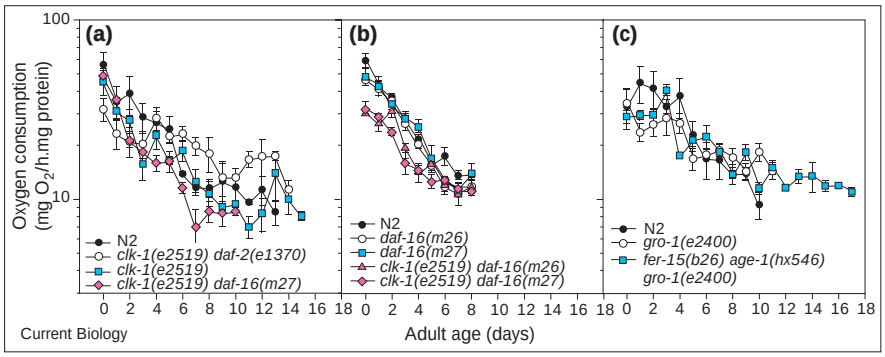
Results and discussion

The oxygen consumption rates of the mutant strains and the wild-type control strain N2 are compared in Figure 1. During the first 4–5 days of their adult lives, both *clk-1(e2519)* and *gro-1(e2400)* mutant worms had respiration rates that were lower than N2 (two-way ANOVA, $p = 0.019$ and 0.034, respectively). As the worms grew older, the difference with the N2 strain became non-significant, because the age-dependent decline of oxygen consumption rates was less steep in the Clk mutants. The double mutant strains *clk-1(e2519) daf-16(m26)* and *clk-1(e2519) daf-16(m27)* consumed less oxygen than either of the *daf-16* control strains or N2, suggesting that the reduction in respiratory activity caused by mutation in the *clk-1* gene was not suppressed by mutation in *daf-16* (Figure 1b). The strain *fer-15(b26) age-1(hx546) gro-1(e2400)*, which also carries a life-extending mutation in the gene *age-1*, showed essentially similar (two-way ANOVA, $p > 0.05$) respiration rates as *gro-1* single mutants of the same age, even though the *age-1* mutants lived longer. The simultaneous presence of the life-extending mutation *daf-2(e1370)* significantly attenuated the age-dependent decline of oxygen consumption rates in *clk-1* single mutants, however (F-test for equality among slopes, $p = 0.015$).

The light-production assay, not unexpectedly, generally confirmed these observations, and showed that the Clk mutants had higher metabolic capacities at advanced age relative to the N2 control (Figure 2). Interestingly, luminescence intensity, unlike oxygen consumption, was not reduced in *clk-1 daf-16* double mutant worms relative to the respective *daf-16* controls (Figures 1b,2b). No conclusions can be made about the epistasis of mutation in *daf-16* to *clk-1* because of the weak effect of *clk-1(e2519)* on the light-production potential and scatter in the data. The *daf-16(m26)* strain had a higher metabolic potential than *daf-16(m27)* over the first 4 days of adult life, but no allele-specific difference was observed in the respective double mutants with *clk-1*. The aberrant depression in luminescence intensity produced by 4–8-day-old *fer-15 age-1 gro-1* mutant worms is out of keeping with correlative anomalies in the survival, respiration rate and ATP profiles of this strain, and, therefore, probably has no biological relevance.

In the wild-type control, ATP stores decreased by about eightfold as the worms grew very old. Quite surprisingly, ATP reserves decreased little during the entire adult life time of both the *clk-1(e2519)* strain (F-test for equality among slopes, $p = 0.0026$) and the *gro-1(e2400)* strain ($p = 0.0020$), relative to N2. The *clk-1(e2519) daf-2(e1370)* and *gro-1(e2400) age-1(hx546) fer-15(b26)* genotypes had increased ATP reserves relative to the single mutants

Figure 1



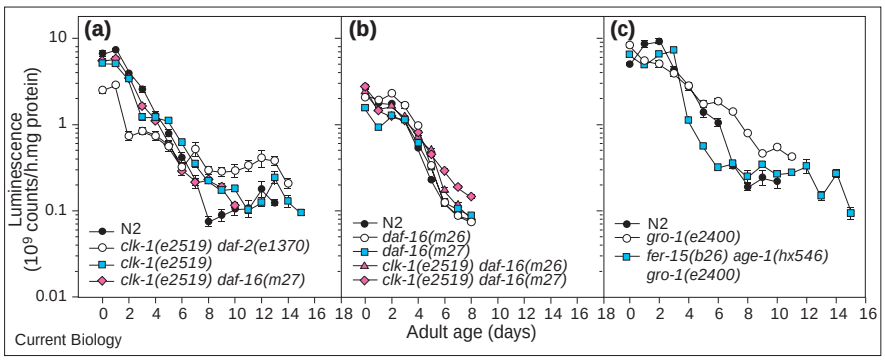
Respiration rates of (a,b) *clk-1* and (c) *gro-1* mutant strains and the control strains N2, *daf-16(m26)* and *daf-16(m27)*. The study cohorts in each panel were initiated simultaneously to reduce environmental variability. Because it takes up to 3 days more for the slowest growers (*gro-1*) to reach adulthood, time is expressed as adult age, day 0 being defined as the first day of adulthood. Respiratory activity declined nearly exponentially with age.

(two-way ANOVA, $p < 0.001$) that remained nearly constant throughout the adult life span. The effect of mutation in *clk-1* was completely suppressed by mutation in *daf-16* (Figure 3).

The idea that metabolic rate is inversely proportional to life span (that is, the 'rate of living' hypothesis) has a long history. It was proposed by Pearl [5] to account for the inverse relationship between ambient temperature and life span in *Drosophila*. Metabolic rate is also inversely correlated with body size and life span in a series of mammals [6], and an intuitive link to a process of wear and tear as an underlying cause of loss of homeostasis and death is then readily made. The now widely accepted idea that endogenous reactive oxygen species (ROS) are likely to be a major cause of ageing and death [7,8] is also often considered as support for the rate of living hypothesis, and Sohal [9] updated the theory to account for the balance between damage by products of oxidative metabolism and the cellular capacity for prevention and repair.

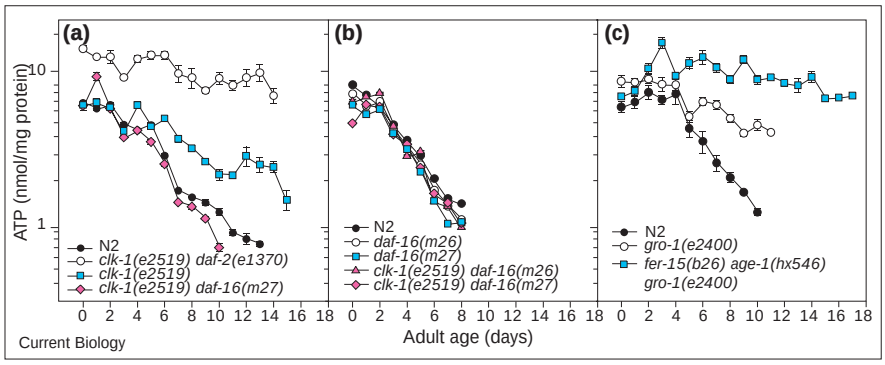
We have shown previously that the oxygen consumption rates were initially essentially similar in adult wild-type and *age-1(hx542)* mutant worms (*age-1(hx542)* is possibly identical with *hx546*, the allele used in this study), but decreased less steeply with age in the Age mutant [10]. Aged *age-1(hx542)* and *daf-2(e1370)* mutant worms retained substantially higher light production capacities than N2 [4,10,11]. The *daf-2* gene (which encodes a member of the insulin receptor kinase family [12]) and the *age-1* gene (which encodes a homologue of the mammalian phosphatidylinositol 3-kinase catalytic subunit [13]) participate in a signal transduction cascade that requires the activity of the downstream *daf-16* gene (encoding a transcription factor of the forkhead family [14,15]) and affects both longevity and dauer formation. There has been some controversy as to whether *daf-16* also suppresses *clk-1*. For example, Lakowski and Hekimi [1,16] reported that mutation in *daf-16(m26)* failed to suppress the long life conferred by mutation in any of the Clk strains, whereas Murakami and Johnson [17] found essentially similar adult life spans for N2, *daf-16(m26)*, *daf-16(m27)* and doubles of either of these *daf-16* alleles with *clk-1(e2519)*. We have included a cohort comprising N2, both *daf-16* mutant strains and the respective doubles with *clk-1* (Figures 1b,2b,3b) to investigate potential epistasis effects involving the respiration rate, metabolic potential and ATP phenotypes. Both *daf-16* alleles produced essentially similar respiration and ATP profiles (Figures 1,3); *daf-16(m26)* could produce more light than *daf-16(m27)* up to the fourth day of its adult life, but this difference disappeared at later ages and might perhaps

Figure 2



Light production capacities of (a,b) *clk-1* and (c) *gro-1* mutant strains and the control strains. The data are for the same cohorts used for the respiration assays. Light production capacities decreased rapidly with age over the first 8–10 days of adult life but tended to stabilise as the worms grew older.

Figure 3



ATP concentration in (a,b) *clk-1* and (c) *gro-1* mutant strains and control strains. The data are for the same cohorts used for respiration and light production assays. ATP stores decreased nearly exponentially with age after an initial period of 2–4 days of adult life. The age-dependent decreases in the *clk-1 daf-16* double mutants were similar to those in the respective *daf-16* controls and almost similar to N2 (a,b), but low in genotypes with mutation in *clk-1* or *gro-1*, and nearly absent in the *clk-1 daf-2* and *gro-1 age-1 fer-15* strains.

not be relevant. Most intriguingly both alleles suppressed the ATP phenotypes imparted by mutation in *clk-1*, but failed to suppress the reduction of respiratory activity. Neither allele was able to suppress the inability of *clk-1(e2519)* to mature in standard axenic medium. When autoclaved *Escherichia coli* cells were added to this medium, *clk-1(e2519)* mutants matured and lived about twice as long as N2. The Age phenotype was greatly suppressed in *clk-1(e2519) daf-16(m27)* double mutants (Table 1). Thus mutation in *daf-16* can suppress some but not all phenotypes caused by mutation in *clk-1*.

Of the four Clk genes only *clk-1* has been cloned. The predicted gene product is a homologue of yeast Cat5p/Coq7p [2,18,19], which is a mitochondrial protein directly involved in the biosynthesis of ubiquinone [20]. The mild reduction of oxygen consumption by young adult *clk-1* mutant worms is compatible with an anticipated reduction of ubiquinone concentration in the mitochondria of these worms. Our experimental results also demonstrate, however, that ATP production cannot be seriously compromised in the Clk mutants and suggest that energy consumption is to some extent uncoupled from energy production in these worms. This would explain both the extraordinary high levels of ATP and the slowing down of the pace of many cellular and organic functions in these mutants.

The weaker age-dependent declines of respiration rate and metabolic potential combined with the Age phenotypes of the Clk mutants can be understood as a consequence of slowing of biological time relative to chronological time. Helfand *et al.* [21] used an elegant approach to distinguish expression patterns linked to biological rather than to chronological time by plotting changes of expression against the percentage maximum life span. This approach was not possible in this study because accidental progeny, even though very scarce, would preclude maximum life span determination in cultures comprising several hundreds of thousands of worms. One could anticipate, however, that this approach would produce steeper plots of metabolic activity against life span, such that increases in metabolic activity in older mutant animals, relative to wild type, might disappear.

In conclusion, our experimental results indicate that metabolic rate is reduced in the young adult Clk mutants, although the capacity for ATP generation is not compromised. Probably energy production and consumption are largely uncoupled in these mutants. The fact that mutation in *daf-16* suppressed both the Age and ATP phenotypes, but not the oxygen consumption rate phenotypes of *clk-1*, indicates that the Clk Age phenotype does not result from reduction in oxidative consumption, and casts doubt on the assumption that the increased life span in the Clk mutants would be directly linked to a rate of living effect.

Table 1

Life span of *C. elegans* in axenic medium.

				Lifespan (days)		
Strain	Exp	FUdR	N	Max	Mean	SE
N2	1	+	213	41	26.7	0.4
daf-16(m27)	1	+	210	38	23.9	0.4
N2	2	+	64	48	30.9	0.8
clk-1(e2519)	2	–	60	78	63.4	1.4
clk1(e2519) daf-16(m27)	2	–	59	53	35.9	1.0

Experiments (Exp) 1 and 2 refer to synchronised age cohorts. The axenic medium consisted of 3% soy-peptone, 3% yeast extract and 0.5 mg/ml haemoglobin. Autoclaved *E. coli* cells were added at 5×10^9 cells/ml to all cultures as the *clk-1* mutant fails to mature in standard axenic medium. 5-fluorodeoxyuridine (FUdR) was added at 50 μg/ml to prevent progeny production. The strains harbouring *clk-1(e2519)* matured in this medium but failed to reproduce. N indicates the number of worms analysed. SE indicates standard error of the mean.

Materials and methods

The cultures were initiated from embryonating eggs as described [4,10]. Pre-adult (mostly L4) larvae were washed off the plates and suspended in S buffer (0.1 M NaCl, 50 mM potassium phosphate buffer pH 6.0) containing 10 μg/ml cholesterol, 50 μM FUdR (to suppress reproduction) and approximately 5×10^9 *E. coli* cells per ml, at worm densities not exceeding 1500 per ml. Portions of 200 ml suspension culture were transferred to Fernbach flasks, and continuously shaken at 120 cycles/min. The incubation temperature was 24°C. Bacterial cells were added as needed to replace consumed cells. Samples were harvested mostly at daily intervals. The worms were allowed to settle by unit gravity force, and cleaned by floatation on dense sucrose [22] except that 40% w/w sucrose solutions were used. Dead worms were removed by centrifugation through 36% (v/v) Percoll in S buffer at 1000 rpm for 2 min [23]. This procedure was repeated, if necessary, to obtain preparations that contained over 95% live worms. Sampling was discontinued if this specification could not be met any more. Oxygen consumption was measured using a Clark electrode as described [10]. ATP concentrations were determined using the standard luciferin/luciferase assay. A commercial kit (Boehringer Mannheim) was used following the manufacturer's instructions. Frozen nematode samples were boiled for 15 min to kill endogenous ATPases instantly. This treatment also resulted in efficient extraction of ATP. Reactions were carried out in white microtiter plates, and the light emitted was monitored using the Victor 1420 Multilabel Counter from Wallac (Turku, Finland).

We have amended our light production assay to fit the microtiter plate format. Briefly, 15 μl suspended worms (8 replicate assays per sample) was pipetted into the wells of a white microtiter plate and the reaction was started by adding 75 μl complete lucigenin cocktail (containing 0.1 mM lucigenin, 0.2 mM NADH, 0.2 mM NADPH, 5 mM KCN). Chemiluminescence intensity was recorded over 60 min at 25°C. The time integrals were determined automatically and used for expressing oxidative capacities. In our previous work [4,10,11] the light production potential (capacity) was expressed as peak chemiluminescence intensity because of technical limitations. We have scaled our measurements of respiration rate, metabolic potential and ATP concentrations against protein content [4]. A different picture would be obtained if these parameters of metabolic activity were scaled against the number of worms assayed, because the average individual mass differed among the various genotypes and tended to fluctuate over time. In our experience, variance of worm mass is also difficult to control rigidly within one experiment and even more so among different experiments.

Supplementary material

A supplementary figure showing the effects of the various mutations or mutation combinations on life span is published with this paper on the internet.

Acknowledgements

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Supplementary material

Apparent uncoupling of energy production and consumption in long-lived Clk mutants of *Caenorhabditis elegans*

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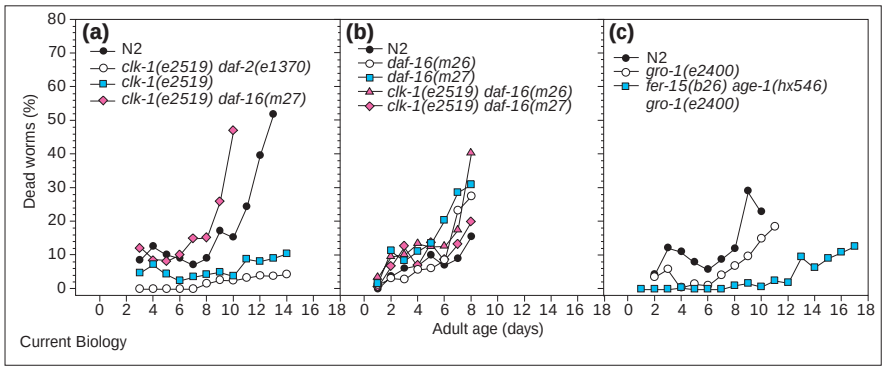
Figure S1



The fraction of dead worms in the ageing (a,b) *clk-1* and (c) *gro-1* cohorts used for the various assays. The study cohorts in each panel were initiated simultaneously to reduce environmental variability. Because it takes up to 3 days more for the slowest growers (*gro-1*) to reach adulthood, time is expressed as adult age, day 0 being defined as the first day of adulthood. The apparent depression of dead worms during the first week of adult life correlates with repeated transfers to fresh culture medium. Live worms collect more rapidly at the bottom of the flasks; a fraction of dead worms is then aspirated together with the old medium.