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Metabolism, physiology and stress defense in three aging Ins/IGF-1 mutants of the nematode *Caenorhabditis elegans*

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Summary

The insulin/insulin-like growth factor-1 (Ins/IGF-1) pathway regulates the aging rate of the nematode *Caenorhabditis elegans*. We describe other features of the three Ins/IGF-1 mutants *daf-2*, *age-1* and *aap-1*. We show that the investigated Ins/IGF-1 mutants all have a reduced body volume, reduced reproductive capacity, increased ATP concentrations and an elevated stress resistance. We also observed that heat production is lower in these mutants, although the respiration rate was similar or higher compared with wild-type individuals, suggesting a metabolic shift in these mutants.

Key words: aging; *C. elegans*; Ins/IGF-1 signaling; longevity; metabolism; stress defense.

Introduction

Lifespan, development, stress resistance and metabolism of the nematode *Caenorhabditis elegans* are regulated by an endocrine pathway sharing homology with the insulin and insulin-like growth factor-1 (IGF-1) pathways in mammals (Kenyon *et al*., 1993; Lithgow *et al*., 1994, 1995; Riddle & Albert, 1997; Kenyon, 2001; Pierce *et al*., 2001; Partridge & Gems, 2002; Houthoofd *et al*., 2005). The activity of the pathway is regulated by insulin-like ligands that bind to the Ins/IGF-1 receptor DAF-2. Activation of the receptor results in autophosphorylation and subsequently the phosphorylation of the PI-3-kinase heterodimer, which is composed of the regulatory p85 subunit AAP-1 and the catalytic p110 subunit AGE-1. The PIP_2 and/or PIP_3 formed recruit AKT-1, AKT-2, SGK-1 and PDK-1 to the plasma membrane where PDK-1 activates the AKT and SGK-1 kinases by phosphorylation (Alessi *et al*., 1997; Hertweck *et al*., 2004). The three kinases can antagonize the FOXO transcription factor DAF-16 by phosphorylation, but the AKT proteins preferentially regulate dauer formation whereas SGK-1 is the crucial factor for the control of development, stress response and longevity (Hertweck *et al*., 2004). In individuals undergoing stress, lacking food-sensing amphids or germ line cells, or carrying a mutation in one of the genes encoding proteins active in the Ins/IGF-1 pathway, DAF-16 is found in the nucleus, whereas under normal growth conditions, DAF-16 resides in the cytoplasm (Henderson & Johnson, 2001; Lin *et al*., 2001).

It has often been assumed that the increased metabolic activity boosted by Ins/IGF-1 signaling under conditions that favor rapid growth and reproduction would shorten life by virtue of an increased generation of reactive oxygen species (ROS) thought to cause aging (Harman, 1956). The central idea is that mitochondria are particularly susceptible to oxidative damage as they produce substantial amounts of free radicals as byproducts of normal metabolic activity. In support of this concept, several mutants of *C. elegans* with impaired mitochondrial function are long lived. A crucial question is whether the association of impaired mitochondrial function and prolonged lifespan results from fewer ROS being produced by reduced metabolic rates or from increased protection from ROS or both. Respiration rates in *clk-1* mutants, a gene involved in the synthesis of ubiquinone (Ewbank *et al*., 1997; Jonassen *et al*., 2001; Rea, 2001), and in *gro-1*, a gene involved in the fidelity of mitochondrial translation (Lemieux *et al*., 2001), are not widely different from wild-type (Braeckman *et al*., 2002a), but mutants in *isp-1*, which encodes an iron–sulphur protein of complex III, are hypometabolic (Feng *et al*., 2001). RNAi knock-downs of *nuo-2* (a component of complex I), *cyc-1* (a component of complex III) and *cco-1* (a component of complex III) show reduced rates of respiration and ATP production. However, restoring messenger RNA to normal levels during adulthood did not elevate ATP or respiration rate, suggesting that mitochondrial function early in life establishes rates of respiration behavior and aging that persist during adulthood and that hypometabolism *per se* is not responsible for these effects (Dillin *et al*., 2002). This finding is consistent with recent studies reporting that high aerobic respiration is not necessarily linked to increasing free radical generation. Indeed, at low rates of respiration the mitochondrial inner membrane potential is very high, resulting in high ROS generation. Membrane potential drops as respiration intensifies, releasing less ROS (Korshunov *et al*., 1997; Brand, 2000; Nicholls, 2002). Alternatively, increased protection from ROS might

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underlie the prolonged lifespan of mutants with impaired mitochondrial function. This explanation was proposed for the *isp-1* mutants (Feng *et al*., 2001). However, Lee *et al*. (2003) found that RNAi inactivation of NADH cytochrome Q oxidoreductase B18 (complex II), ubiquinol-cytochrome b reductase (complex III), cytochrome c oxidase Viic (complex IV), cytochrome c oxidase Vb (complex IV), cytochrome c oxidase IV (complex IV), mitochondrial ribosomal subunit, cytochrome c heme lyase and two mitochondrial carriers enhanced resistance to hydrogen peroxide but not to paraquat. Thus the mechanism by which impaired mitochondria extend lifespan appears to be more complex than simply increasing global defense against ROS.

We have previously reported that *daf-2(e1370)* mutants dissipated less heat per unit oxygen consumed and had elevated levels of ATP relative to wild-type worms. In addition, homogenates of *daf-2(e1370)* displayed higher SOD and catalase activities and they were able to reduce the dye 2,3-bis (2methoxy-4nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxyanilide (XTT) faster than did homogenates obtained from wild-type worms (Houthoofd *et al*., 2005). It is likely that reduced Ins/IGF-1 signaling causes these effects. However, other explanations cannot be excluded, e.g. these effects might be allele-specific, or they might be mediated by a distinct pathway emanating from DAF-2 and not involving PI-3-kinase. Compelling evidence has been advanced supporting the existence of such a parallel signaling pathway (Gems *et al*., 1998; Wolkow *et al*., 2002; Hertweck *et al*., 2004).

To investigate these possibilities we measured the effects of the *aap-1(m889)* and *age-1(mg305)* mutants in addition to *daf-2(e1370)* on physiology, metabolism and stress defense. *m889* is the only allele of *aap-1* identified thus far. It likely causes a severe defect in AAP-1 because it truncates the protein after the first of two SHC boxes (Wolkow *et al*., 2002). *daf-2(e1370)* and *age-1(mg305)* animals share a number of strong phenotypes including exceptional longevity and sensitivity to high osmolarity. The *mg305* allele was also chosen as a result of an ongoing investigation (K.H. and A. Wolkow, unpublished results) of the effect of tissue-specific expression of *age-1* transgenes on the physiology, metabolism and stress defense of *C. elegans*.

Results

Body size, reproductive capacity and lifespan

Wild-type animals continued to grow in body length and body width for the first 3–4 days of adulthood and showed few, if any, changes in size at later ages (Fig. 1A–C). The Ins/IGF-1 mutants stopped growing 2 days earlier. The size of *aap-1* animals remained essentially invariant thereafter, but *daf-2* and *age-1* animals shortened substantially with increasing age. In all, the Ins/IGF-1 mutants had body volumes that were reduced by 32–42% between days 4 and 10 of adulthood. The brood size of *daf-2(e1370)*, *age-1(mg305)* and *aap-1(m889)* animals was reduced by 72, 98 and 30%, respectively, independent of the growing temperature (Table 1). Average lifespan was increased by 60, 297 and 188%, respectively (Fig. 1D).

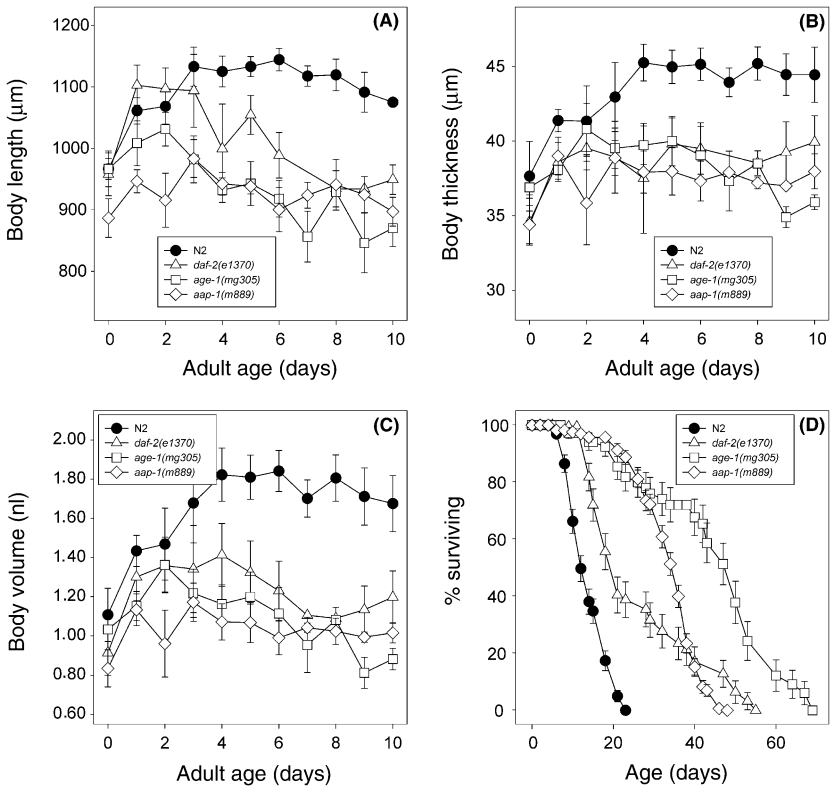

Fig. 1 Body sizes and lifespan of wild-type (N2), *daf-2*(*e1370*), *age-1*(*mg305*) and *aap-1*(*m889*) animals as a function of age. (A) Body length; (B) body width; (C) body volume; (D) survival of the four strains at 22.5 °C. Average lifespan (days) ± SE (*n*) are: N2: 11.9 ± 0.5 (121), *daf-2*(*e1370*): 19.1 ± 1.3 (200), *age-1*(*mg305*): 47.3 ± 1.6 (200), *aap-1*(*m889*): 34.2 ± 0.7 (180).

Table 1 Brood size of wild-type and Ins/IGF-1 mutants. Worms were grown at 20 or 16 °C until L4 and subsequently shifted to 22.5 or 25 °C to prevent dauer formation. Brood size was decreased substantially at both growing temperatures, in accordance with earlier results (Gems *et al.*, 1998)

Strain	20→22.5 °C	%N2	16→25 °C	%N2
N2	241 ± 39	100	156 ± 19	100
daf-2(e1370)	64 ± 21	27	47 ± 12	30
age-1(mg305)	6 ± 9	3	3 ± 9	2
aap-1(m889)	170 ± 32	71	107 ± 20	69

Metabolic rate

We examined the metabolic rate of the Ins/IGF-1 mutants by measuring respiration rate and heat production. Oxygen consumption declined steadily with age in N2 animals but much less so in *daf-2(e1370)* and *aap-1(mg305)* animals. Consequently, the mutant worms consumed more oxygen than did wild-type from day 3 onwards (Fig. 2A; $P < 0.003$). Unfortunately, we were not able to determine respiration of *age-1(mg305)* animals because the size of the aging cohort was prohibitively small, owing to the low fertility of this strain. Heat dissipation measurements are more sensitive and could be performed for the *age-1* animals as well. All three Ins/IGF-1 mutants released less heat during the first 4 days (Fig. 2B; $P < 0.02$), but the difference disappeared as the worms grew older. Thus, the heat production rate did not follow the respiration rate. This causes a different calorimetric/respirometric (C/R) ratio, which is a measure of catabolic efficiency (Kemp & Guan, 1997). *daf-2* and *aap-1* mutants have a lower C/R ratio compared with wild-type worms during most of the adult life (Fig. 2C; $P < 0.05$ for the age class 2–6 days of adult age). A still more pronounced difference was reported for *daf-2(e1370)* previously (Houthoofd *et al.*, 2005).

ATP content

It is generally assumed that ATP production rate and ATP consumption rate are tightly coupled, resulting in a constant ATP content. However, the results shown in Fig. 3(A) indicate that this is not the case: first, ATP concentrations steadily decline with age in wild-type worms, resulting in a 50% decrease every 4.5 days. Secondly, the three investigated mutations also influence the ATP content of worms. The mutants have increased ATP contents from day 1 of adult life onwards (*t*-test: $P < 0.05$) and the ATP content does not show an age-dependent decrease in these strains from day 3 onwards (Fig. 3A; *F*-test: $P > 0.1$).

XTT-reduction capacity

Whereas XTT reduction capacity declined steadily with age in wild-type animals, no such decline was seen in any of the Ins/IGF-1 mutants (Fig. 3B; *F*-test: $P > 0.5$). This effect causes a higher reduction in capacity during most of the adult life of the

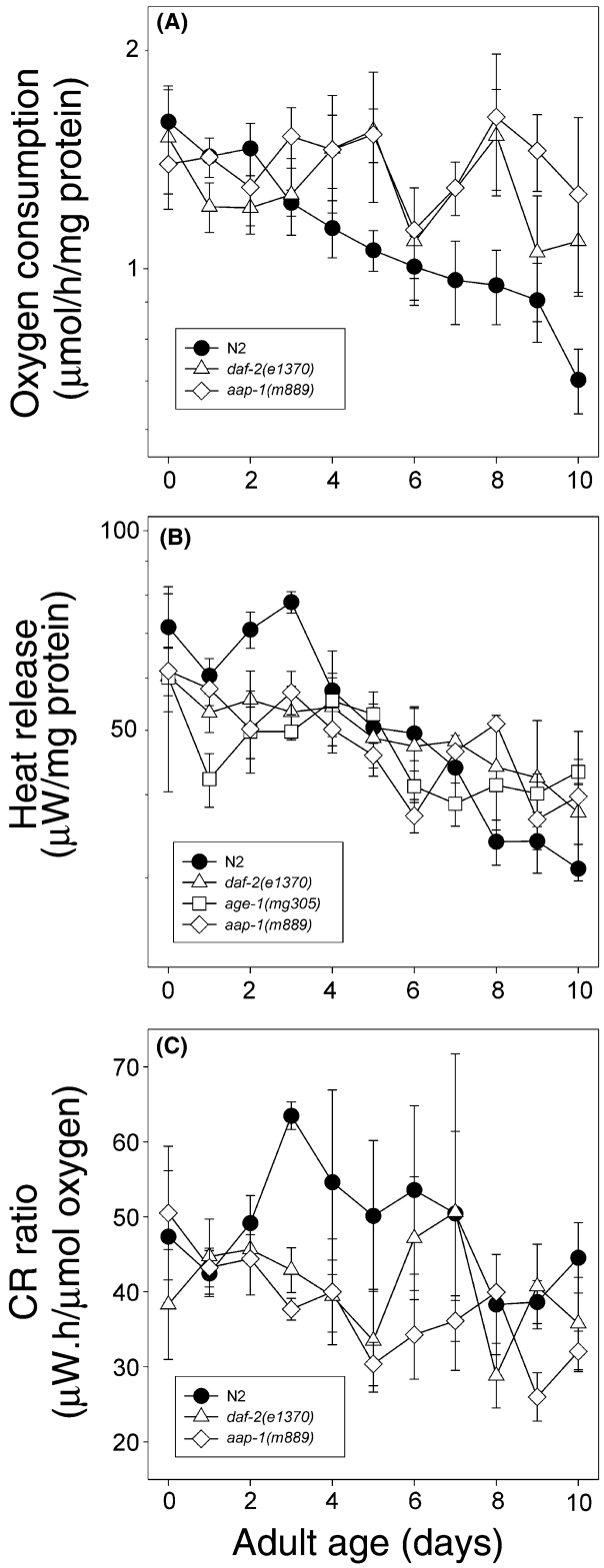

Fig. 2 Metabolic rate and C/R ratio of aging wild-type and Ins/IGF-1 mutants. (A) Respiration rate; (B) heat production; (C) C/R ratio.

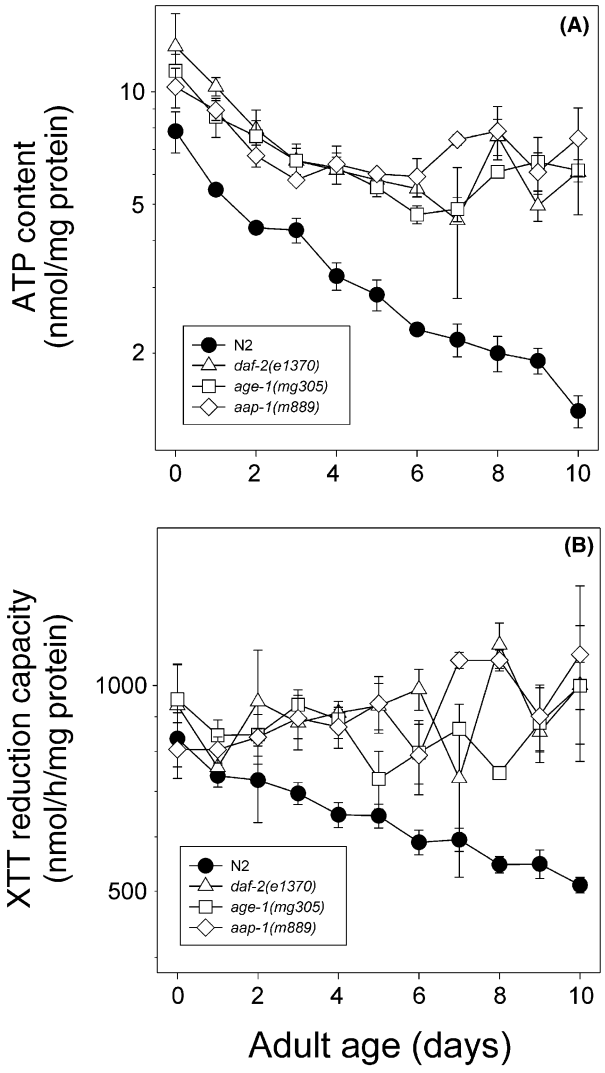

Fig. 3 (A) ATP concentration in wild-type and the three Ins/IGF-1 mutants during the adult life trajectory. (B) XTT reduction capacity in the four strains during the aging process.

Ins/IGF-1 mutants. The XTT reduction rates decreased by about 75% in all strains when 30 U mL^{-1} SOD from bovine erythrocytes (Sigma, St Louis, MO, USA) was added to the reaction mixture (data not shown), indicating that enzymatic reductase activity and superoxide accounted for approximately 25 and 75% of the XTT reduction, respectively.

Stress defense

Reduction of Ins/IGF-1 signaling enhanced the resistance to elevated temperature. Interestingly, *age-1(mg305)*, the longest lived mutant strain under non-stressed conditions, exhibited superior resistance to elevated temperature (Fig. 4A). All three Ins/IGF-1 mutants had SOD activity levels that were about twice as high as those measured in wild-type animals (Fig. 4B; $P < 0.001$). Catalase activity was also elevated in all three mutant strains (Fig. 4C; $P < 0.001$).

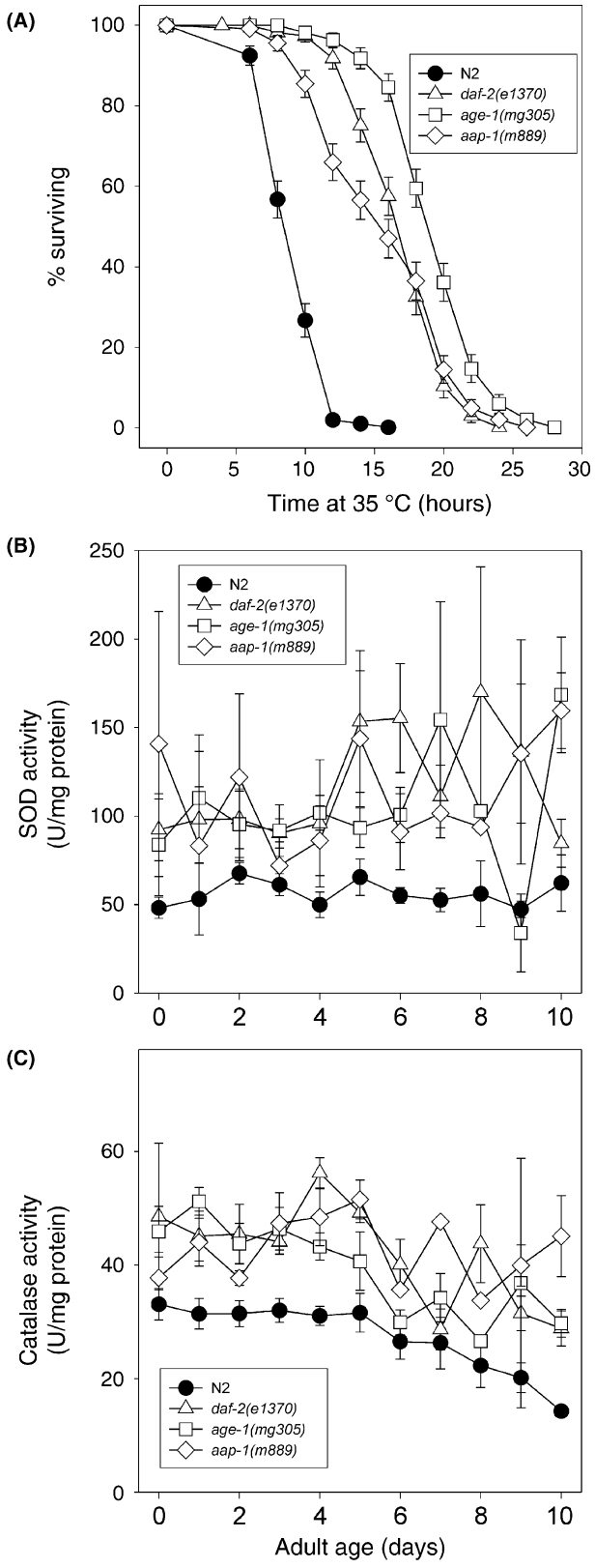

Fig. 4 (A) Resistance to elevated temperature (35 °C) of wild-type and Ins/IGF-1 mutants. Average survival time (hours) ± SE (*n*) are: N2: 8.4 ± 0.3 (120), *daf-2(e1370)*: 16.6 ± 0.4 (120), *age-1(mg305)*: 18.8 ± 0.4 (120), *aap-1(m889)*: 15.4 ± 1.0 (120). (B) SOD activity in aging wild-type and Ins/IGF-1 mutants. (C) Catalase activity in the four strains during the aging process.

Discussion

We found that all three Ins/IGF-1 mutants had a reduced body size relative to wild-type animals. Van Voorhies & Ward (1999) also found smaller body sizes for *daf-2(e1370)* animals, but McCulloch & Gems (2003) reported larger body sizes for 1-day-old *daf-2(e1370)* adults. A number of methodological differences can be invoked to explain this discrepancy. McCulloch and Gems maintained the worms on agar with a lawn of *Escherichia coli* cells as a food source whereas in our study the worms were maintained in liquid culture consisting of *E. coli* in S medium.

Differences in culture temperature, however, are likely to have a greater impact. We raised the worms on agar plates at 17 °C until they had reached the fourth larval stage and transferred them to liquid culture at 24 °C, whereas McCulloch and Gems maintained the cultures invariably at 15 °C. *daf-2(e1370)* animals are nearly wild-type at low temperature but are dauer-constitutive and exhibit several other defects, including a severe reduction of brood size, at high temperature. The animals in the latter study therefore conceivably contained more eggs and perhaps more germ cells. On the other hand, it should be noticed that gravid *aap-1* animals had smaller body volumes than *daf-2* and *age-1* worms, although they contained many more eggs. It is also possible that the shift to the restrictive temperature attenuated any further increase in body size. It should also be mentioned that body volume was inferred from measurements of body volume and width, treating worms as cylinders in both studies. Body widths are very small and differences in defining the borders are bound to result in exponentially divergent estimates of volume, from calculations based on the square of the radius. Finally swelling or shrinkage artifacts associated with the measuring techniques might also contribute to the discrepancy between the McCulloch and Gems study and our measurements. Indeed, worms with mutation in the Ins/IGF-1 pathway were found to be specifically sensitive to high osmolarity (J. Wang, personal communication). The issue of body size is important because a reduced body size in all three Ins/IGF-1 mutants would indicate that Ins/IGF-1 signaling promotes body size in *C. elegans*, as it does in *Drosophila* (Böhni *et al*., 1999; Weinkove & Leevers, 2000; Garofalo, 2002) and rodents (Brown-Borg *et al*., 1996; Coschigano *et al*., 2000; Flurkey *et al*., 2001). Unfortunately, all the caveats just mentioned weaken this conclusion.

It has long been known that whole organism metabolic rate scales with a power function (b) of body mass ($1 - b$ for mass-specific metabolic rate). There is still much dispute about the precise numerical value of b. The scaling exponent $b = 2/3$ originally proposed by Max Rubner (1908) is consistent with the rate at which heat produced within a volume can be dissipated through the surface. Recently, White & Seymour (2003) provided support for a scaling exponent of $b = 2/3$ from an experimental study encompassing 619 species from 19 mammalian orders. Kleiber (1947) and Brody (1945), however, measured a greater exponent of about 3/4 in a series of mammals. Quarter-power scaling has since been based on theoretical grounds and regarded as applicable to poikilotherms and homoiotherms, single cells and even subcellular organelles as well (West *et al*., 1997, 1999, 2002; Gillooly *et al*., 2001). In the most recent attempt to resolve this argument, Demetrius (2003) postulated that energy transduction in a biological organism is constrained by processes linked to the coupling of electron transport and proton translocation, and constraints imposed by ecological and demographic forces. The mathematical outcome is that the scaling exponent will follow a 3/4 rule in the case of organisms subject to scarce but dependable resources and a 2/3 rule when they are subject to ample but only temporarily available resources, i.e. both exponents are applicable, depending on the natural history of the species. In the case of *C. elegans*, the 2/3 rule seems theoretically more appropriate. In the absence of compelling evidence for any numerical value of the scaling exponent, we have used the scaling exponent $b = 0.72$, which was derived empirically from a series of 68 nematode species (Klekowski *et al*., 1972).

We have monitored respiration repeatedly in worms carrying a mutation in *daf-2* or *age-1* and found that the rates of oxygen consumption were either similar or higher (Vanfleteren & De Vreese, 1996; Braeckman *et al*., 2002c; Houthoofd *et al*., 2005; this study), but never lower, in these mutants relative to the wild-type. Methodological differences may explain the contradictory results with Van Voorhies & Ward (1999). First, Van Voorhies & Ward (1999) ignored size differences. Second, these authors measured CO_2 production, and the amounts of CO_2 released per O_2 consumed may be different in mutants that exhibit reduced Ins/IGF-1 signaling. SAGE analysis of dauer vs. non-dauer transcripts (Holt & Riddle, 2003) and measurements of enzymatic activities in dauers and adult worms (O'Riordan & Burnell, 1989) suggest that dauers actively fix CO_2 in phosphoenolpyruvate, forming oxaloacetate for anabolic reactions. In addition, dauers and Ins/IGF-1 mutants have prominent fat reserves and dauers heavily rely on the combustion of stored fat that has a lower CO_2/O_2 ratio relative to carbohydrate. Because entry into the dauer stage depends on down-regulation of Ins/IGF-1 signaling, it is likely that similar metabolic shifts occur in Ins/IGF-1 mutants.

Oxygen consumption and heat production rates are equal descriptors of energy metabolism, and in principle they can be interconverted using an oxycaloric equivalent of –450(± 5%) kJ mol^{-1} O_2 for all biologically useful substrates (Gnaiger & Kemp, 1990). It should be stressed that our experimental set-up precludes this approach. Respiration and heat production were measured under very divergent environmental conditions. Respiration was assayed immediately after the worms had been cleaned with sucrose and Percoll, and the contents of the measuring cell were required to be rapidly stirred for accurate measurements. In contrast, isothermal microcalorimetry required absence of agitation and at least 1 h of equilibration to reach a stable signal. Thus the animals were excited in one experimental environment and at rest in the other. Nevertheless, we can reasonably assume that the ratio between oxygen consumption and heat output will remain invariant among strains as long as

the assay conditions remain unaltered. The lower C/R ratio is not likely reduced by the reduced number of eggs in the gravid hermaphrodites because we observed no decrease in C/R ratio in sterile *glp-4(bn2)* worms (data not shown). Thus the decrease of the C/R ratio in worms in which Ins/IGF-1 signaling is reduced by mutation points to changes in the behavioral response to the assay conditions, changes in fluxes through alternative pathways of energy metabolism, or to increased efficiency of ATP generation. Rea & Johnson (2003) hypothesized that a shift in the relative contribution of two coexisting metabolic pathways, aerobic respiration and fermentative metabolism, especially fermentative malate dismutation, might be responsible for the increased longevity seen in many animals with impaired Ins/IGF-1 signaling or mitochondrial function, the tenet being that these pathways generate fewer ROS. Several lines of evidence indicate that *C. elegans* can operate to some extent anaerobically. The animals excrete lactate, acetate, succinate and propionate when incubated under anoxic conditions (Föll *et al*., 1990) and dauer larvae and *daf-2* mutants are resistant to hypoxia in an allele-specific manner (Scott *et al*., 2002). Furthermore, *C. elegans* has a functional glyoxylate cyle (O'Riordan & Burnell, 1990; Holt & Riddle, 2003; Murphy *et al*., 2003; Wang & Kim, 2003), and possesses the necessary tools for mitochondrial malate dismutation, including mitochondrial fumarate reductase and rodoquinone (Takamiya *et al*., 1999; Holt & Riddle, 2003), cytosolic malate dismutation, and ethanol and lactate fermentation (Holt & Riddle, 2003; Rea & Johnson, 2003). Any anaerobic heat is predicted to increase the C/R ratio. Yet, we found that the C/R ratio was lower in the Ins/IGF mutants (Braeckman *et al*., 2002c; Houthoofd *et al*., 2005; this paper). Thus it appears that the contribution of anaerobic to total energy metabolism is likely small in the Ins/IGF-1 animals and that they possess a more efficiently operating aerobic energy metabolism.

One possible explanation for the lower C/R ratios in these mutants is that they have lower proton leak rates across the mitochondrial inner membrane and thus produce ATP more efficiently. This might in turn explain the elevated ATP levels measured in these animals. Another possible reason why these mutants generate less heat is that they have reduced anabolic rates. This explanation is not tenable if it is assumed that the endothermic ATP production balances its exothermic hydrolysis. This is true for fully coupled metabolism at steady state where the rate of cellular heat dissipation then measures the rate of the catabolic processes nearly exclusively (Kemp & Guan, 1997). However, the high ATP levels detected in the Ins/IGF-1 mutants indicate that the balance between ATP production and consumption is altered in these animals. These mutants retain higher fat stores and produce fewer offspring, suggesting that they have a reduced energetic demand for anabolic reactions, including the production of considerable quantities of yolk protein (Murphy *et al*., 2003). The increased fat and ATP content in germ-line-deficient mutants also supports this model (K. Houthoofd *et al*., unpublished results). However, the latter hypothesis cannot explain the quite similar ATP levels in the three studied Ins/IGF-1 mutants, which have variably reduced levels of fertility.

XTT reduction capacity did not decrease in the Ins/IGF-1 mutants so that these animals had a higher XTT reduction capacity during most of their adult life. Approximately 75% of this activity is suppressible by exogenous SOD in an age- and genotype-independent fashion. The origin of the superoxide produced in these experiments is unknown, but a respiratory source is unlikely because the homogenates were prepared in the presence of the detergent CHAPS (0.75%). Likely sites of superoxide production under these conditions include dissociated mitochondrial complexes, small pro-oxidants and a variety of redox reactions, especially cytochrome P450-mediated oxidations. The balance of 25% of the XTT reduction capacity that is not suppressible by SOD is likely due to the activity of a number of diaphorases, i.e. enzymes that are capable of oxidizing NAD(P)H by a variety of artificial electron acceptors, including XTT. Interestingly, substantial up-regulation of XTT reduction capacity was also observed for the dauer stage (Houthoofd *et al*., 2002a), consistent with the idea that various metabolic pathways are similarly modulated in the normal dauer stage and in mutants carrying a mutation in the Ins/IGF-1 pathway.

The elevated SOD and catalase activities that we found, including in dauers, corroborate this hypothesis. Up-regulation of catalase and SOD is frequently associated with increased longevity. Increased activities of SOD and catalase were measured in two long-lived *eat-2* mutants, and in wild-type worms grown in axenic medium, which retards aging (Houthoofd *et al*., 2002b,c) and a very large increase was found in *daf-2* mutants that were grown axenically, which correlates with the 6–7-fold increase in lifespan (Houthoofd *et al*., 2003). Yanase *et al*. (2002) and Murphy *et al*. (2003) reported that both *ctl-1* and *ctl-2* are up-regulated in *age-1* and *daf-2* mutants. Reduction of the expression of these genes with RNAi partially suppressed the life extension imparted by mutation in *daf-2* (Murphy *et al*., 2003). *C. elegans* has three genes encoding Cu/ZnSOD, *sod-1*, *sod-4* and ZK430.3 (*sod-5*), and two genes encoding MnSOD, *sod-2* and *sod-3* (http://www.wormbase.org). The manganese-containing enzymes likely contribute a minor fraction of total SOD activity, as little SOD activity survives cyanide poisoning (Vanfleteren, 1993). Expression analysis revealed substantial elevation of *sod-3* and weak up-regulation of *sod-1* in *daf-2* and *age-1* mutants (Honda & Honda, 1999; Yanase *et al*., 2002). Thus at least one isoform each contributing to mitochondrial and cytosolic SOD activity is up-regulated in Ins/IGF-1 mutants. Recently, McElwee *et al*. (2003) reported the intriguing finding that ZK430.3 (*sod-5*) is up-regulated 12.9-fold in a *daf-2 mutant* and that RNAi inhibition of this gene caused a small but significant increase in the lifespan of *daf-2(e1370)* but has no effect on lifespan in a wild-type genetic background.

The simultaneous up-regulation of reducing and detoxifying enzyme activity as inferred from the changes in XTT, and the antioxidant SOD and catalase enzymes may indicate that reduction of Ins/IGF-1 signaling elicits the co-ordinated expression of an elaborate detoxification program, consistent with recent evidence from expression profile analysis (McElwee *et al*., 2004; Gems & McElwee, 2005).

Experimental procedures

Bristol N2 (CGC), *daf-2(e1370)* (CGC), *age-1(mg305)* (C. Wolkow) and *aap-1(m889)* (D. Riddle) were used in this study. *C. elegans* worms were grown and sampled as described in detail previously (Braeckman *et al.*, 2002b; Houthoofd *et al.*, 2002a,b). In brief, worms were cultured at 17 °C on standard agar plates with wild-type *E. coli* as food source until they reached the L4 stage. At that point, they were rinsed from the plates and transferred to Fernbach flasks containing 5×10^9 *E. coli* cells mL^{-1} and 100 μM FUdR. The worm concentration was approximately 1500 mL^{-1} and the flasks were shaken at 120 cycles min^{-1} at 24 °C. The bacterial concentration was kept at a constant level by checking OD_{550} daily and adding *E. coli* cells from a frozen stock. A sample was taken at daily intervals for analysis from the next day (day 0) onwards. Dead worms were removed by differential centrifugation on a Percoll gradient (Fabian & Johnson, 1994) when the fraction of dead worms exceeded 5%. The sample was washed with 40% sucrose to remove bacteria. The source populations were grown on four (mutants) to six (wild-type) different occasions to account for variation caused by inadvertent environmental changes.

Lifespan analysis was performed on NGM plates with *E. coli* strain OP50. Animals were raised at 16 °C until L4 stage at which point the temperature was raised to 22.5 °C. Animals were transferred daily to fresh plates during the egg-laying period and subsequently twice per week. The fraction surviving was scored regularly. For the resistance to elevated temperature, young adults raised at 16 °C were transferred to agar plates (without *E. coli*) at 35 °C and survival was scored at 2-h intervals. Survival curves for both lifespan and thermotolerance were created using the product-limit method of Kaplan & Meier (1958). For brood size assays, ten L4 animals were grown either at 20 or 16 °C and then transferred singly to the appropriate temperature. These animals were placed daily to fresh plates and the number of L1 progeny was counted.

Oxygen consumption rate, heat production, ATP content, XTT reduction capacity, and the activities of SOD and catalase were determined as described previously (Braeckman *et al.*, 2002a; Houthoofd *et al.*, 2002a,b). Oxygen consumption was measured polarographically using a six-channel respirometer from Strathkelvin (Glasgow, UK) equipped with Clark electrodes. We suspended the cleaned worms in axenic medium at 1000–2000 worms mL^{-1} and transferred 1 mL into each respirometer cell. Axenic medium was preferred to buffer solution because it is an appropriate food source. The worm suspensions were maintained at 24 °C and continuously stirred and oxygen concentration was monitored for 10–30 min. Oxygen-saturated water (100% setting: 263.1 μmol O_2 mL^{-1}) and a freshly grown and dense *E. coli* culture (zero setting) were used for electrode calibration.

Metabolic heat was measured by microcalorimetry using the Thermal Activity Monitor (TAM, Biometric, Jarfalla, Sweden). A vial containing 1 mL of worms suspended in axenic medium and a control vial containing axenic medium only were fixed onto the measuring unit and sunk into a water bath maintained at 24 °C. Stable heat flows could be monitored for several hours after approximately 1 h of equilibration. Bacterial growth was suppressed by adding 250 U penicillin and 250 μg streptomycin mL^{-1}.

ATP content was determined by monitoring the amount of light emitted when luciferin reacts with oxygen in the presence of luciferase. Flash frozen worms (100 μL) were immediately submerged in boiling water for 15 min to destroy all ATPase activity and to allow diffusion of ATP out of the bodies. The samples were diluted with HPLC-grade water to lower the concentration of ATP and salts which interfere with the assay, and ATP was determined using the assay kit from Roche Diagnostics. The average light intensity produced between 10 and 40 min after initiation of the reaction was used to calculate the ATP content.

Worms were homogenized using a Mini-Beadbeater (Biospec Products, Bartlesvillk, OK, USA) shaken at 5000 strikes min^{-1} for 1 min. The mixture was made 1% in CHAPS and clarified at 20 800 r.c.f. for 10 min at 4 °C. The capacity of the supernatant fraction to reduce the tetrazolium compound XTT to the formazan derivative was assayed in the presence of 2 mM each of NADH and NADPH (the reduced nicotinamide co-factors are unable to reduce XTT directly). Unidentified NAD(P)H-dependent diaphorases and superoxide generated by metabolic activity in the tissue extract are XTT-reducing agents. XTT-reducing activity was therefore measured in the presence and absence of 30 U mL^{-1} SOD from bovine erythrocytes to distinguish both sources of XTT-reducing activity. The assay was performed in 96-well microtiter plates and changes in absorbance were monitored at 475 nm for 1 h, at 25 °C. A molar extinction coefficient of 16 399.6 cm^{-1} M^{-1} (Paull *et al.*, 1988) was used for conversion to nmol h^{-1} mg^{-1} protein.

Body volume was determined by measuring the length and thickness of 300–500 fixed worms using the RapidVue (Beckman Coulter) and using a cylindrical model for calculation. All metabolic parameters were correlated to protein content to account for differences in body mass. Respiration rate and heat production were also corrected for mass-specific differences according to the Brody–Kleiber equation (Brody, 1945; Kleiber, 1947) using a *b*-value of 0.72 (Klekowski *et al.*, 1972; Braeckman *et al.*, 2002c; see Discussion). Two-way-ANOVA, Student's *t*-test and *F*-test for regression analysis were used for statistical analysis.

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