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Ageing is reversed, and metabolism is reset to young levels in recovering dauer larvae of *C. elegans*

Koen Houthoofd, Bart P. Braeckman, Isabelle Lenaerts, Kristel Brys, Annemie De Vreese, Sylvie Van Eygen, Jacques R. Vanfleteren*

Department of Biology, Ghent University, K.L. Ledeganckstraat 35, B-9000 Ghent, Belgium

Abstract

The nematode *Caenorhabditis elegans* responds to unfavourable environmental conditions by arresting development and entering diapause as a dauer larva. Dauers can survive several times the normal life span and the duration of the dauer state has no effect on postdauer life span. This led to the suggestion that dauers are non-ageing, and that dauers eventually perish as the consequence of depletion of stored nutrients. We have investigated physiological changes associated with long-term diapause survival, and found that dauer larvae slowly develop senescence-like symptoms, including decrease of metabolic capacity, aconitase enzyme activity, and ATP stores, and increase of lipofuscin- and oxidised flavin-specific fluorescence. However, these changes are reversed when the dauers recover. Thus senescence can occur before attainment of reproductive maturity, and furthermore, is reversible. Other life processes, including respiration rate and heat output, remain unaltered over four weeks of diapause at 24 °C. Possible determinants of the enhanced life maintenance include increased resistance to oxidative stress provided by enhanced superoxide dismutase and catalase activities, and a shift to a highly reducing redox status.

Keywords: Ageing; *Caenorhabditis elegans*; Dauer; Biochemistry; Longevity; Life span; Metabolism; Rejuvenation

1. Introduction

C. elegans develops through four larval stages to reach adulthood. Under adverse environmental conditions, such as when food is depleted and population density or temperature is high, second stage larvae may not moult to the regular third stage larva, but enter a developmentally arrested, non-feeding stage, called the dauer larva (Albert and Riddle, 1988; Riddle and Albert, 1997). Dauers have an altered metabolism and can survive several times the normal life span (O'Riordan and Burnell, 1989, 1990). Interestingly, the duration of the dauer state has no effect on postdauer life span. This led to the suggestion that dauers are non-ageing, and that dauers eventually perish as the consequence of depletion of stored nutrients (Klass and Hirsh, 1976). We could not validate this assumption. Instead, we show that dauer larvae do exhibit a number of symptoms of senescence, but at a reduced rate, and that these are reversed when dauers recover and resume development. Thus senescence can occur before attainment of reproductive maturity, and furthermore, is reversible. Elevated activities of SOD and catalase, and increased reductive capacity also subside upon dauer larva recovery

* Corresponding author. Tel.: +32-9-264-5212; fax: +32-9-264-8793.

E-mail address: jacques.vanfleteren@rug.ac.be (J.R. Vanfleteren).

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2. Materials and methods

Animals: The wild type strain (N2) of *C. elegans* was used and maintained at 24 °C throughout this study (Braeckman et al., 2002; Sulston and Hodgkin, 1988). Synchronous populations were initiated from eggs prepared by alkaline hypochlorite treatment of gravid adults (Fabian and Johnson, 1994) and grown on nutrient agar plates containing a lawn of freshly grown *E. coli* 9001 cells. Samples were taken for the various biochemical assays after 0(unfed L1), 6(L1), 12(L1), 18(L2), 24(L2), 30(L3), 36(L4), 42(L4) and 48 (young adult) hours. After 38 h, a portion of the plates was harvested and the worms were further grown at low population densities (approx. 2000/ml) in S buffer and a relatively constant *E. coli* concentration of 3×10^9 cells/ml. The cells were added from a 1:1 v/v suspension in S buffer, pH 6.0 that was dripped in liquid nitrogen and stored at −75 °C. When the worms reached the fourth juvenile stage FUdR was added at 50 (suspension cultures) or 400 μM (plate cultures) final concentration to prevent progeny production. Live *E. coli* cells consume and degrade FUdR, necessitating approximately 8-fold higher concentration of FUdR to prevent reproduction on plate cultures, which contain a lawn of bacteria. Exposure to 400 μM FUdR did not affect adult morphology, behaviour or longevity of *C. elegans* on plates. Dauers were grown by spreading 150,000 eggs, 10^{10} heat killed *E. coli* cells and 1 mg haemoglobin (from a 5% stock solution in 0.1 N KOH, autoclaved for 10 min) on 10 cm agar (made up with S buffer, pH 7.0 and cholesterol) plates. These conditions induce almost 100% dauer formation. Immediately after dauers had formed, a portion of the plates was harvested and these dauers were transferred into Fernbach flasks containing 200 ml S buffer (pH 7.0), and shaken at 120 cycles per minute at 24 °C. Samples were taken at regular time intervals for the various assays. Dead worms, owing to their slightly lower gravity, were removed by centrifugation through 36% (v/v) Percoll (Fabian and Johnson, 1994), and debris and remaining bacteria were removed by floatation on 40% sucrose (Sulston and Hodgkin, 1988). Cleaned worm samples were examined microscopically to assess whether bacterial contamination was negligible and could be ignored. Harvesting was discontinued when the percentage of dead worms in the cleaned sample began to exceed 5%. Dauers occasionally recovered on the plates with time. Plates containing less than 99% dauers were discarded. Forced recovery after diapause periods lasting for 6 and 27 days was achieved by transferring washed dauers onto nutrient agar (GIBCO) plates with freshly grown *E. coli* cells. Dauer survival was near 100% up to 28 days. Metabolism during recovery was monitored by sampling every 3 h, for a total of 12 h. For investigating postdauer metabolism, postdauer L4 juveniles were further grown in liquid culture as described.

Assays: ATP, oxygen consumption and light production potential were assayed as previously described (Braeckman et al., 2002). Heat output was measured with a thermal activity monitor (Biometric, Jarfalla, Sweden). Adults grown on plates were also assayed on an agar surface containing a lawn of autoclaved *E. coli* cells; those grown in liquid culture were suspended in axenic medium (3% soy-peptone, 3% yeast extract, 0.5 mg/ml haemoglobin), which supports sustained energy expenditure. Antibiotics (250 U/ml penicillin and 0.25 mg/ml streptomycin) were added to prevent interference by bacterial growth for many hours. Dauers were assayed in S buffer or on agar made up in S buffer, because the axenic medium strongly promotes dauer exit.

Bioreduction capacity was measured by adding 5 μl homogenate to a mixture containing 0.4 mM NADH, 0.4 mM NADPH and 0.2 mg/ml XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] (Goodwin et al., 1995). The reduction rate was measured as the increase of absorption against time at 475 nm at 25 °C. Homogenate was prepared by violently shaking (Mini Bead Beater, Biospec Products, Bartlesville, OK) a suspension of worms in 50 mM Na/K phosphate buffer pH 7.0 and glass beads during 1 min. The homogenate was made 1% in CHAPS (3-[(3-Cholamidopropyl)dimethylammonio]-1-propane-sulfonate, Sigma) to dissolve membrane lipids and clarified by centrifugation at 14,000 rpm for 8 min. Autofluorescence was measured with the following absorption/emitting wavelengths (nm):B (355/460) and F1 (450/535). Catalase (Aebi, 1984), SOD (Corbisier et al., 1987) and aconitase (Das et al., 2001) activities were assayed at 25 °C, essentially according to existing protocols, adapted for use in microtiter plate format.

All parameters were scaled to total protein amount (Braeckman et al., 2002) and body volume to account for size differences. Body volumes were measured using an image analysis system (IBAS, Kontron). Normalising to volume instead of protein content would increase all dauer activities by some 50% → 20%, relative to young adult → senescent worms (results not shown). The metabolic measurements were mostly repeated at least three times to reduce assay variation. The source populations consisting of age-synchronised worms were grown 3–6 times at varying time intervals to reduce inadvertent environmental fluctuation. Statistical analyses were performed using algorithms implied in Excel 2000 and SPSS.10.

3. Results and discussion

Longitudinal measurements of oxygen consumption rates normalised to protein content are presented in Fig. 1(a). Respiration is highest in the juvenile stages but falls steeply as the worms mature, and declines exponentially with age throughout adulthood, as previously observed (Vanfleteren and De Vreese, 1996) (lack-of-fit test for linearity after log transformation, solid culture $F_{9,30} = 0.892$, $p = 0.544$; liquid culture $F_{12,35} = 0.962$, $p = 0.502$). Dauer larvae consume substantially less oxygen than other stages, and, unlike adults, can maintain essentially constant respiration rates over time. Recovery from the dauer stage is accompanied by a rapid increase of respiratory activity. Interestingly, the duration of the dauer state has little if any effect on postdauer respiratory activity. The oxygen consumption profiles upon recovery after 1 or 4 weeks of diapause are indistinguishable (two-way ANOVA, $F_{1,52} = 0.010$, $p = 0.921$) suggesting that postdauer energy expenditure is not affected by the duration of the preceding diapause. In addition similar age-specific declines of respiration rates are seen in postdauer adults and adults that bypassed the dauer stage (F-test for equality of slopes of several regression lines, $F_{3,46} = 0.901$, $p = 0.448$).

According to the laws of thermodynamics, living systems must release energy, usually heat, to their environment to compensate for the increase in order they create. The heat released varies with metabolic activity and can be accurately measured as heat flow in heat conduction calorimeters. Both catabolic and anabolic pathways dissipate heat, but it has been shown that the contribution by anabolism is negligible (Kemp and Guan, 1997). Since glycolysis appears to be quite low at normoxia in *C. elegans* (Föll et al., 1999), it is not surprising that the heat output and respiration profiles in Fig. 1(a) and (b) are generally similar. There is one notable difference in that dauers raised on plates, and assayed on an agar surface, dissipate more heat than those in suspension culture (Fig. 1(b)). This is a likely response to the more adequate oxygen supply when the worms are exposed to a gaseous environment. The respiration profiles shown in Fig. 1(a) do not reveal this effect, probably since respiration could only be measured in liquid medium.

The ATP profiles (Fig. 1(c)) resemble those obtained for oxygen consumption and heat production. ATP levels are not exceptionally lower in dauers relative to the other juvenile stages. These findings contrast a previous study reporting that the phosphorous (^{31}P) NMR signal corresponding to ATP was not detectable in dauer larva extracts (Wadsworth and Riddle, 1989). Unlike respiration rates and heat output, dauer ATP concentrations decrease gradually with age. Could this decline result from progressive exhaustion of stored nutrient supplies? Two observations suggest not. First, ATP levels decrease exponentially with time (lack-of-fit test for linearity after log transformation, solid culture $F_{5,26} = 0.482$, $p = 0.787$; liquid culture $F_{4,20} = 0.968$, $p = 0.447$), from the very beginning onwards. It is common knowledge that ATP is held at fairly constant levels as long as combustible fuels, mainly fat and carbohydrate, are not depleted. Thus, if fuel provisions were involved, one would expect no changes in ATP levels initially, but only later on, when these fall beyond a critical level. Secondly, the respiration and heat dissipation rates remain unchanged over the entire time span suggesting that the nutrient stores are adequate. Thus we favour the conclusion that the declining ATP levels result from declining mitochondrial function. Gradual increases in mitochondrial dysfunction occur with age (Floyd et al., 2001). This view is also corroborated by the age-dependent declining capacity for metabolic activity in adults and dauers. Metabolic capacity was measured using a

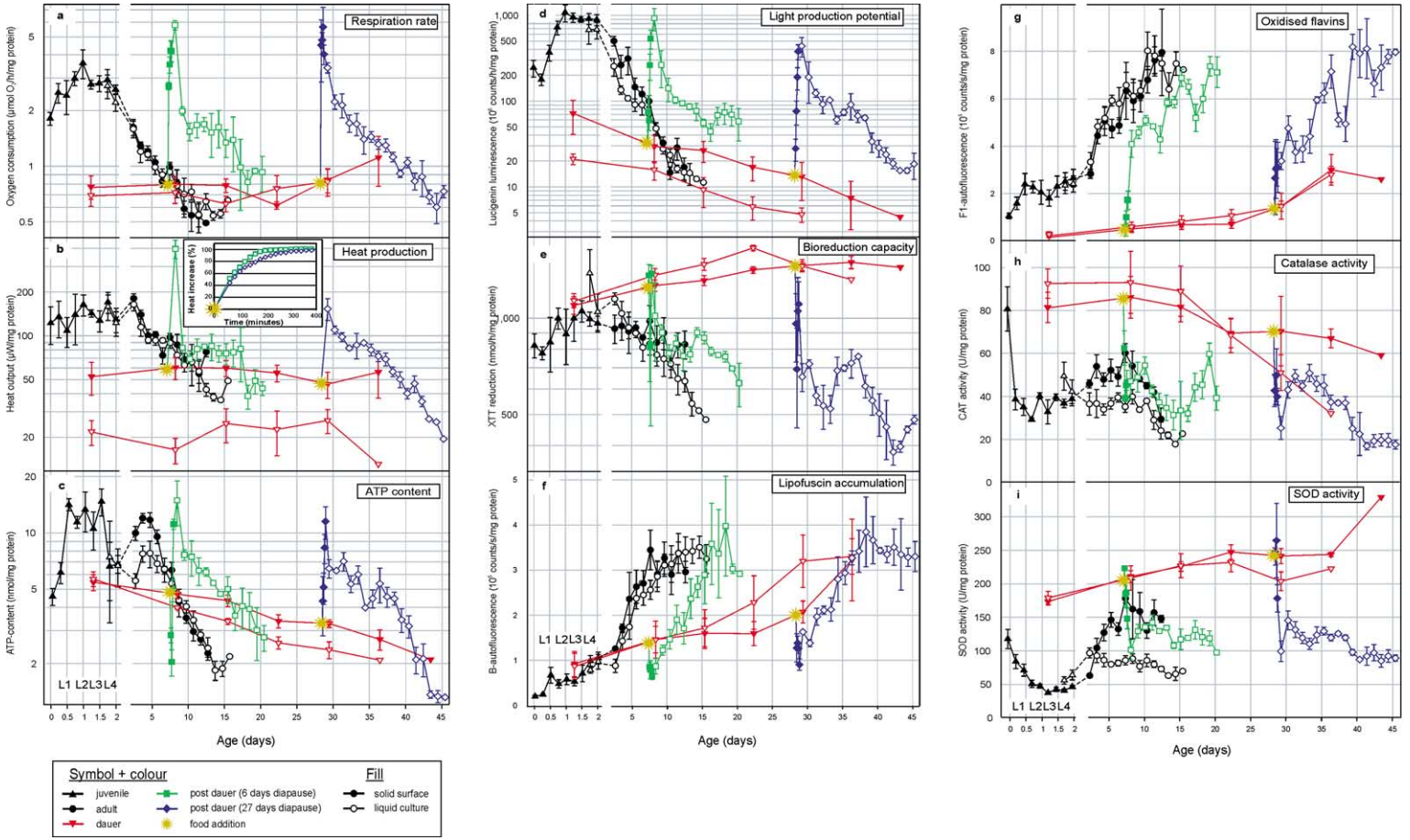

Fig. 1. Metabolic and enzyme activity profiles as a function of development and ageing. Full lines indicate consecutive sampling of source populations; dotted lines connecting symbols indicate that the data correspond to separate experimental populations. The *y*-axis for respiration, heat production, ATP, light production potential, and XTT reduction capacity is in the log scale. Real time heat increase during dauer recovery is shown as an insert to (b). The relatively long time for stabilisation (30–60 min) and the fast rise of heat production during recovery precludes monitoring of heat production by means of consecutive sampling. L1, L2, L3, L4: first, second, third and fourth larval stage. The error bars denote S.E.M.

light production potential (LPP) assay (Vanfleteren and De Vreese, 1995, 1996; Braeckman et al., 2002) (Fig. 1(d)). Light is produced when freeze-thawed worms are suspended in assay medium containing lucigenin, NADH, NADPH and KCN. The nicotinamide coenzymes fuel reactions that produce superoxide, which in turn reacts with lucigenin to produce an excited product that emits a photon when it disintegrates. Cyanide is added to block Cu/ZnSOD, and cytochrome oxidase, thus enhancing mitochondrial superoxide production. It is estimated that 1–3% of the oxygen taken up by animals is converted to superoxide in normally functioning mitochondria (Halliwell and Gutteridge, 1999). Thus any change in maximum mitochondrial output will be highlighted in the LPP assay.

The LPP profiles reveal several features. As previously reported (Vanfleteren and De Vreese, 1996; Braeckman et al., 1999), the metabolic capacity is highest in the L2–L4 stages and declines exponentially and substantially (approx. 20-fold over 2 weeks of adult life) with age in the adults. Dauers exhibit reduced metabolic capacities and these also decrease exponentially with time (approx. 5-fold over 4 weeks), and in the long run they fall to very low levels. The low LPP levels in dauers may result in part from increase of SOD-3, a MnSOD isoform upregulated in dauers (Honda and Honda, 1999). As mentioned above we assume that the decline of LPP with time reflects progressive reduction of mitochondrial performance. Recovery from the dauer stage is accompanied by a rapid increase of the LPP, reflecting a steep increase in mitochondrial capacity.

Whereas direct NAD(P)H-dependent reduction of the tetrazolium salt XTT to its water soluble formazan derivative is negligible in the absence of appropriate intermediate electron acceptors such as phenazine methosulfate, we found that addition of nematode extract boosted rates of XTT reduction by 1–2 orders of magnitude in a linear dose dependent relationship (Braeckman et al., 2002). Approximately 50% of this activity is suppressed by exogenously added superoxide dismutase (SOD), suggesting that it is due to superoxide (results not shown), and we infer that the activity that is not suppressible by SOD is contributed by unknown NAD(P)H dependent reductase(s). By using this assay we can monitor changes in XTT bioreduction capacity that occur as a function of the life cycle trajectory. In adults, the potential to reduce XTT declines quickly with age (Fig. 1(e)). Dauers, however, exhibit substantially enhanced reduction capacity, which tends to further increase during the first 2–3 weeks of diapause and then stabilises. The upregulation of XTT reduction capacity is quickly abolished as the dauer larvae commit to recovery.

Age pigment (lipofuscin) and reduced nicotinamides emit blue fluorescence at 420–470 nm when excited with wavelengths in the range of 344–370 nm (Davis et al., 1982), however, nicotinamide fluorescence constitutes a very minor portion of the signal (unpublished observations). Thus we infer that the fluorescence intensities shown in Fig. 1(f) correspond to a rapid increase of lipofuscin with age in adults. Age pigment also accumulates with time in dauers (regression analysis, solid culture: $\beta_1 = 0.687$, $P < 0.001$; liquid culture: $\beta_1 = 0.753$, $P < 0.001$) and perhaps even faster in dauers maintained in liquid culture suggesting that dauers do senesce. Fluorescence emitted by oxidised flavins also increases steeply in ageing adults and more gradually in dauers (Fig. 1(g)). This signal is quite low in dauers. Most likely this is due to a shift to the reduced form, consistent with the fast reversal accompanying dauer exit, rather than a general decrease in flavin content. Standard tests for estimating oxidative damage accumulation such as the thiobarbituric acid and carbonyl assays (Halliwell and Gutteridge, 1999) produced signals that were hardly above background in senescent worms, and suffered from large variance (results not shown), so they were considered not useful for assessing age-related damage in dauers. However, aconitase activity decreased by 50–60% in 4-week-old dauers (one-way ANOVA, solid culture: $F_{6,27} = 3.293$, $p = 0.015$; liquid culture: $F_{5,21} = 8.509$, $P < 0.001$; Fig. 2). Aconitase is particularly sensitive to oxidative damage during ageing due to carbonylation (Yan et al., 1997; Das et al., 2001; Gardner and Fridovich, 1991) and direct inactivation of the 4Fe–4S cluster by superoxide (Gardner and Fridovich, 1991).

Catalase (Fig. 1(h)) and SOD (Fig. 1(i)) activities are substantially upregulated in dauers confirming previous observations (Vanfleteren and De Vreese, 1995; Anderson, 1982; Larsen, 1993), and decline sharply after exposure to food to stabilise at the levels normally seen in adults. Adults grown on a solid

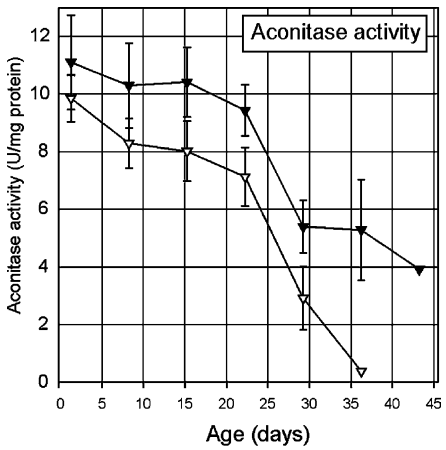

Fig. 2. Aconitase activity in dauers. Full symbols: dauers maintained on agar plates; open symbols: dauers in liquid culture. Error bars denote S.E.M.

surface have generally higher enzyme activities relative to those in liquid culture. Perhaps this is an adaptive response to the 30-fold higher concentration of oxygen in air.

So, what makes dauers long-lived? A popular theory is the rate-of-living theory. It assumes that organisms have a predetermined metabolic potential i.e. they can only spend a predetermined amount of energy, expressed as total amount of oxygen consumed per unit body weight, during the life span. A major implication is that life span is inversely proportional to metabolic rate (Pearl, 1928). This prediction is also underpinned by the more sophisticated oxidative-damage theory (Harman, 1956). However, the oxygen consumption profiles observed for adult worms that bypassed, and those succeeding, the dauer stage are quite similar, even after 4 weeks of dauer diapause. Thus life expectancy is not determined by the rate of living, and other mechanisms of life span determination must be involved. Our present results confirm increased resistance to oxidative stress as a well-known key factor determining dauer longevity. Both SOD and catalase activities are upregulated in dauers. However, juveniles exhibit higher metabolic rates (Fig. 1(a) and (b)) but lower activity levels of SOD and catalase relative to the adults, and they do not exhibit faster rates of lipofuscin accumulation (Fig. 1(f)). Thus oxidative stress is not the only cause of ageing. The possibility that the high XTT reduction activity in dauers is due to superoxide, is unlikely on the basis of the elevated SOD activity in dauers. Increased enzyme reductase activity is a more likely cause, and taken together with the low oxidised flavin content may indicate a shift to a more negative oxidation–reduction potential in dauers, but further work will be needed to validate this idea.

Do dauers senesce? The oxygen consumption and heat output rates suggest not. On the other hand, gradual declines of metabolic capacity, aconitase activity, and ATP stores, and increases of age pigment and oxidised flavins suggest that dauers do not completely resist ageing. As discussed above we favour the idea that the decreases in ATP and LPP result from age-dependent declining mitochondrial function. How can stable oxygen and heat dissipation rates, observed in dauers, be reconciled with the postulated declining mitochondrial capacity? The former activities occur in living cells, whereas the LPP assay measures maximal superoxide production rates (typically 1–3% of the oxygen consumed) in freeze-thawed and thus variably uncoupled mitochondria, fed with non-limiting amounts of respiratory substrate (NADH), which is directly available to the electron transport chain. Thus declines in mitochondrial capacity are likely to precede signs of declining function. This hypothesis is also corroborated by the absence of any visible effect of the age-dependent losses of aconitase activity on oxygen consumption and heat dissipation rates in dauers. The steep increase in LPP, which accompanies the exit of diapause, should then point to either rapid repair, or increase in size, or a burst of mitochondrial divisions to replace damaged mitochondria. Thus mitochondrial ageing might occur during dauer diapause, albeit slowly and not compromising the potential for mitochondrial rejuvenation, when commitment to dauer exit is made.

Since all ageing-like changes rapidly disappear when dauers recover, with no visible effect on postdauer life, it must be concluded that dauers can reverse ageing and reset the clock when they recover.

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