The Effect of Dietary Restriction on Mitochondrial Protein Density and Flight Muscle Mitochondrial Morphology in *Drosophila*

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Dietary restriction (DR) extends life span in diverse organisms and may do so by attenuating production of mitochondrial reactive oxygen species (ROS). However, measurements of ROS production from isolated mitochondria of organisms subjected to DR have produced inconsistent results. In the fruit fly *Drosophila*, DR does not reduce production of ROS from isolated mitochondria. In this study, we used *Drosophila* to test whether DR lowered mitochondrial density. We assessed mitochondrial densities of flies on DR and Control diets using (a) the activities of mitochondrial enzymes and (b) electron microscopy. Both methods showed no overall effect of DR on mitochondrial density; however, mitochondrial enzyme activities and morphology differed significantly between DR and Control flies. We concluded that life-span extension by DR in *Drosophila* is not mediated through a reduction in mitochondrial density.

If DR in *Drosophila* extends life span by reducing ROS production, then it does so through mechanisms that operate only in vivo.

**The free radical (mitochondrial) theory of aging** (1,2) proposes that mitochondria play a central role in the aging process by virtue of being a major source of reactive oxygen species (ROS). If this theory were correct, then variation of ROS production or antioxidant defense systems would be predicted to modulate life span. Correlational support for the mitochondrial theory of aging comes mainly from the finding that rates of mitochondrial ROS production vary inversely with maximum life-span potential of several animal species (3–7). Comparative studies of ROS production and maximal life span between birds and mammals of similar body size have shown that birds are longer-lived possibly because they exhibit lower rates of mitochondrial ROS production compared to mammals (7,8). Studies of *Drosophila* have also shown that longer lived strains contain higher levels of cytosolic copper-zinc superoxide dismutase (CuZnSOD) compared to shorter lived strains (9). Genetic manipulations of various antioxidant enzymes have provided additional support for the role of ROS in limiting life span. Knockout of the mitochondrial manganese superoxide dismutase (MnSOD) gene in mice leads to severely shortened life span (10), and silencing of the same gene in *Drosophila* leads to early adult-onset mortality (11). However, gene knockouts are known also to introduce novel pathologies to the organism, but it is inconclusive whether they normally limit life span. Overexpression of antioxidant defenses has, however, produced variable effects. Whereas targeted overexpression of CuZnSOD in *Drosophila* motor neurons was shown to extend life span by up to 40% (12,13), other studies involving overexpression of the same enzyme in flies have shown that life-span extension can be influenced by genetic background (14).

Dietary restriction (DR) is an environmental intervention whose life-prolonging effects have been observed in diverse animal species (15,16). One of the suggested mechanisms of action of DR is through decreasing mitochondrial ROS production and the associated oxidative damage (17–20). However, the effect of DR on limiting ROS production in isolated mitochondria appears inconsistent given the disparate findings from different researchers. In rodents, DR was shown to decrease mitochondrial generation of hydrogen peroxide (21–24) as well as the accumulation of oxidative damage to protein (25,26), lipids (27), and DNA (24,28,29). However, other researchers have observed no effect of DR on mitochondrial ROS production. In *Drosophila*, Miwa and colleagues (30) did not find any differences in mitochondrial ROS production between DR and fully fed controls, and similar observations were later made with cells isolated from rats (31). The inconsistent effects of DR in rats on mitochondrial ROS production measured in isolated mitochondria in vitro or in state 4 resting condition (in the absence of adenosine 5′-diphosphate [ADP]) and in cells suggest that conditions for isolated mitochondria may not adequately mimic mitochondrial ROS production in vivo where ADP and other factors such as a regulated fuel supply are present.

Another way in which DR could lower ROS production in vivo is by reducing the mitochondrial density (MD).
would lower the number of ROS-producing sites and make each mitochondrion carry out oxidative phosphorylation at a higher rate, lowering its proton motive force and therefore its ROS production (32,33). In this study, we tested whether DR decreases MD in vivo using the Drosophila model. Drosophila is an organism well suited for this purpose because it shows life-span extension in response to DR (34,35) similar to rodents, and is easier to handle and manipulate under laboratory conditions. Determination of MD was carried out using two methods: 1) indirectly, by measuring the activities of mitochondrial enzymes (matrix: citrate synthase [CTS] and MnSOD; inner membrane: cytochrome c oxidase [COX] and adenine nucleotide translocase [ANT]) in isolated mitochondria compared to homogenates; and 2) directly, by using electron microscopy (EM) of flight muscle mitochondria. The enzyme method relies on the assumption that cell populations (hence mitochondrial populations) harvested from the two groups of flies are the same, whereas the stereological method used in EM can be prone to bias (36). Because each method has its own strengths and weaknesses, it is advantageous to use both. The enzyme method also gives additional information about intrinsic properties of the mitochondria such as the activities of oxidative phosphorylation and antioxidant enzymes, whereas the EM method can additionally provide information on mitochondrial morphology such as shape and volume. The aim of this study therefore was to investigate whether life-span extension by DR involved changes in MD or other properties of the mitochondria.

Methods

Chemical Reagents

The following reagents and biochemicals from Sigma Chemical Company (St. Louis, MO) were used: imidazole; ethylenediamine tetraacetic acid (EDTA); 5,5'-dithiodisulfone-2-nitrobenzoic acid (DTNB); acetyl-coenzyme A (acetyl-CoA); oxaloacetate, oxidized cytochrome c; Tween-80; Triton X-100; potassium cyanide (KCN); rotenone; oligomycin; carbonyl cyanide p-(trifluoromethoxy)phenylhydrazone (FCCP); P4,P3-di(adenosine-5') pentaphosphate; L-α-glycerophosphate; bovine serum albumin (BSA); ethylene glycol-bis(2-aminoethyl ether)-N,N',N''-tetraacetic acid (EGTA); xanthine; nitroblue tetrazolium (NBT); sodium cyanide; diethylenetriaminepentaaetic acid (DTPA); catalase; bathocuproine disulphonic acid; and xanthine oxidase. Carboxyatractysoside (CAT) was obtained from Calbiochem (Nottingham, U.K., Ltd). All other chemicals and reagents were of pure analytical grade.

Experimental Flies

Male Drosophila melanogaster were used for enzyme measurements to avoid complications from ovarian activity, while female flies were used for flight muscle EM because of their larger size and greater ease of handling. All flies were of the wild type Dahomey base stock, and both sexes increase life span in response to DR (35). For the enzyme experiments, flies were cultured for two generations on normal strength 10% sucrose–yeast (SY) medium in 189-ml bottles at standard density (37). Flies of uniform age were collected for the experiments from the third generation using light CO2 anesthesia. A total of 6000 flies (3000 Control and 3000 DR) were set up for each of the four enzyme assays. For EM, eggs were collected from stock cages by introducing petri dishes containing grape agar medium (30% grape juice, 2.5% agar, 0.2% nipagin) seeded with a small amount of live yeast. The plates were retrieved 4 hours later and incubated at 25°C overnight. The next day, first instar larvae were picked from the plates and placed in vials of 10% SY medium at 50 larvae per vial. When the flies had emerged, they were placed in vials of fresh SY food for 48 hours. Females were then collected under CO2 anesthesia and placed on experimental food.

DR

The concentrations of yeast and sugar in the DR medium was 4% for enzyme assays and 6.5% for EM [the concentrations that maximize Dahomey male and female life span, respectively (35,38)] and that in the Control medium was 16% and 15%, respectively. Flies were randomly allocated to each of the DR and Control regimens in groups of 100 flies per 189 ml bottle (10 flies per 7 ml vial for EM), and were transferred to fresh food once every 3 days (3 times per week for EM). Deaths were scored daily.

Flies for the enzyme assays and EM studies were collected from randomly selected bottles or vials, and the required numbers were counted under CO2 anesthesia. Sampling of flies was done at comparable chronological and physiological ages for the DR and Control groups. The time points for the enzyme assays were those corresponding to survivorship of 95%, 65%, and 35% for the Control flies and additional sampling points were introduced when survivorship for the DR flies had reached 65% and 35%. For the EM experiments, samples were taken for dissection at 80% and 60% survivorship for the Control group as well as at time points when DR survivorship had reached 80% and 60% survivorship.

Enzyme Assays

Whole-fly homogenates were prepared from 10 flies in the appropriate buffer for each of the enzymes CTS, COX, and MnSOD. For the CTS assay, the buffer was 50 mM imidazole containing 2 mM EDTA and 0.5% Triton X-100, pH 7.1 (Buffer A); for the COX assay, it was 200 mM Tris-HCl containing 0.03% Tween 80 pH 7.5 (Buffer B); for MnSOD, it was 50 mM potassium phosphate, pH 7.8 (Buffer C). The flies were chilled on ice, weighed, and homogenized in 1 ml of buffer on ice by using three 20-second bursts of a T8 Ultra-Turrax homogenizer (Jencons-PLS) given at 1-minute intervals. The homogenates were centrifuged twice at 2000 g for 15 seconds in a refrigerated centrifuge (4°C), and the resultant supernatant were assayed immediately for enzyme activity.

Mitochondria were isolated according to procedures described earlier (33) in freshly prepared STE buffer (250 mM sucrose, 5 mM Tris, 2 mM EGTA, and 1% BSA, pH 7.1). Mitochondrial pellets for the CTS, COX, and MnSOD assays were dissolved in the appropriate assay buffer to about 30 mg/ml. For the ANT assay, mitochondrial pellets
were gently resuspended in 0.5 ml of STE buffer (25–30 mg protein/ml) taking care to avoid rupturing the mitochondria. Fly mitochondria become uncoupled quickly (33), thus all procedures were carried out speedily on ice (or 4°C) and the mitochondria were used within 1 hour of isolation.

Protein determinations in homogenates and mitochondrial fractions were carried out using the Pierce BCA Protein Assay Kit (Perbio Science, UK Ltd., Cheshire). Enzyme activities for CTS, COX, and MnSOD were assayed under conditions of enzyme saturation with substrate in a HITACHI model U-2001 spectrophotometer (Hitachi High-Technologies Corporation, Wokingham, U.K.) at 25°C, and ANT activity was measured using a Clarke-type Electrode (Rank Brothers, Cambridge, U.K.). The enzyme activities were expressed as both activity per mg protein and as activity per fly. The concentrations of reagents given for all assays are final concentrations unless stated otherwise.

CTS activity was measured in fresh whole-fly homogenates and fresh mitochondrial fractions using the procedure described by García-Esquível and colleagues (39) with modifications. The reaction mixture contained 50 mM Buffer A, 0.1 mM DTNB, 0.3 mM acetyl-CoA, 0.05 mM oxaloacetate, and 10 μL of whole-fly homogenate or mitochondrial fraction. The reaction was initiated by addition of the oxaloacetate (omitted from the blank), and the reduction of DTNB was monitored at 412 nm.

COX activity was measured according to the method of Smith (40) with modifications. Enzyme activity was measured in a cuvette containing Buffer B, 0.03% Tween-80, 100 μM reduced cytochrome c, and 10 μL of whole-fly homogenate or mitochondrial protein. The oxidation of reduced cytochrome c was followed at 520 nm in a spectrophotometer at 25°C. The background (nonenzymatic) rate was determined by adding 200 μM cyanide, and did not exceed 2%–5% of the enzymatic rate under these assay conditions.

MnSOD activity was determined using the procedure described by Mockett and colleagues (41) with some modifications. The whole-fly homogenates and mitochondrial fractions were first passed through a Sephadex G-25 column to remove small-molecular-weight antixidants that could interfere with the assay (42). The eluate was incubated with 5 mM KCN at room temperature for 45 minutes to completely inhibit the cytosolic CuZnSOD. The activity of the whole-fly homogenate or mitochondrial fractions to inhibit the xanthine/xanthine oxidase-driven autoxidation of NBT in Buffer C was monitored at 560 nm (41). The unit of MnSOD activity was calculated as the amount of protein that gave half-maximal inhibition of NBT oxidation under the assay conditions.

ANT activity was measured as follows: To the thermoregulated (25°C) Clarke-type electrode chamber were added 1.95 ml of KHE buffer (120 mM KCl, 5 mM KH2PO4, 3 mM HEPES, 1 mM EGTA, 1 mM MgCl2, and 0.2% BSA [pH 7.2]), 10 μL of 1 mM rotenone, 20 μL of 6 mM P4P5, di(adenosine-5')pentaphosphate, and whole-fly homogenate or mitochondrial fraction at about 0.1–0.2 mg/ml; then the chamber was sealed to exclude atmospheric air. While measuring respiration, 20 μL of 2 M α-glycerophosphate and 10 μL of 50 mM ADP were added to obtain states II and III respiration rates, respectively. The reaction was then titrated with aliquots of 10 μM stock of CAT until the respiration rate decreased to the state II rate. Finally, 20 μL of 5 mM FCCP were added to ensure that the mitochondria were still coupled; otherwise, fresh mitochondrial isolates had to be prepared. ANT activity was calculated as the extrapolated minimum amount of CAT needed to fully return respiration to state II from a plot of respiratory rate versus CAT concentration, and was expressed as picomoles of CAT titer per mg protein or per fly.

Mitochondrial Protein Density

The MD of DR and Control flies was calculated indirectly from the activities of enzymes in whole-fly homogenates and mitochondrial fractions per mg protein. The whole-fly homogenate enzyme activity was divided by the mitochondrial enzyme activity to give mg mitochondrial protein per mg whole-fly homogenate.

EM and Stereology

Flies were dissected for examination of flight muscle by transmission EM in Drosophila Ringers solution (43). The indirect flight muscles and the dorsal longitudinal muscles (DLMs), were examined [reviewed in (44)]. Heads and abdomens were removed, and the thoraces were bisected longitudinally along the midline to expose the flight muscle. The thorax halves were then placed in primary fix (3% gluteraldehyde, 2.5 mM CaCl2, 100 mM HEPES (pH 7.2), H2O2 added to 0.33% just before use) at 4°C for 3 hours (45) after which they were washed with a solution containing 2.5 mM CaCl2 and 100 mM HEPES (pH 7.2) four times, each wash lasting 15 minutes. They were then put in secondary fix [1% osmium tetroxide, 1.5 % ferricyanate, 100 mM HEPES (pH 7.2) (46)] for 1.5 hours and then washed in distilled water four times for 15 minutes each time before storage at 4°C.

Dehydration of samples was done by first washing several times with increasing concentrations of ethanol, then incubating in two changes of acetonitrile, and finally embedding in Spurr’s resin. The samples were then cured at 60°C for 24 hours. Sections were cut at 50 nm in the transverse plane for volume fraction estimates or rotated to produce a vertical section plane for surface-density estimates with a Leica Ultracut UCT microtome (Leica, Vienna, Austria). They were mounted on 300 mesh copper grids, stained with uranyl acetate and lead citrate, and were then viewed on a Philips CM-100 transmission electron microscope operated at 80 Ky (Philips, Eindhoven, The Netherlands).

The mitochondrial volume density (Vv (mt,c)) was measured according to the stereological methods described (36). A square 16-point lattice with a spacing of 17.6 μm was overlaid on the display monitor connected to a Gatan 673 CCD camera (Gatan, U.K.) at a final magnification of ×2800. Fields of view were randomly sampled by identifying the first grid square containing flight muscle, positioning the lattice in the upper right hand corner of the grid (36), and repeating this in subsequent grid squares until 50 fields of view had been counted. Lattice intersections overlying mitochondria and myoplasm (including all other
components of the myofibers) were counted. Mitochondrial volume density was calculated by the ratio of points counted within the mitochondrial outer membrane to those outside mitochondria.

For the mitochondrial inner membrane surface density (Sv (mt,mt)) determination, five individual mitochondria from each group were randomly selected by point counting as above and were photographed at a magnification of ×35,000. The negatives from these photographs were scanned and counts were done by digitally overlaying the quadratic lattice with an effective line spacing of 314.2 nm in Adobe Photoshop 6.0.1 (Copyright © 1989–2001, Adobe Systems Incorporated). Inner membrane surface density was calculated by the formula \( S_v = 2i/L \), where \( i \) is the number of intersections with the line matrix for the membrane of interest, \( P \) is the number of points within the area of interest (reference volume), and \( l/p \) is the line length per point (47).

The outer membrane surface densities of mitochondria per cubic micrometer of mitochondria (Sv (mt,mt) or Sv (mt,mt) mitochondrial outer membrane/myofiber, respectively) were also estimated and expressed as square micrometers of outer membrane per cubic micrometer of mitochondria or myofiber. Ten fields of view were randomly selected as above and were photographed at a final magnification of ×4500. They were overlain with a 16-point quadratic lattice with a spacing of 3.4 μm. Linear intercepts with mitochondrial outer membranes were also counted and the formula \( S_v = 2i/L \) was used to estimate surface density of outer mitochondrial membrane, where \( i \) is number of intercepts and \( L \) is total test line length overlying either mitochondria or myofiber (36,47).

Statistical Analyses

Data sets were compared using JMP statistical data package (version 4.05: [Academic] Copyright © 1989–2001, SAS Institute Inc.). Survival data were analyzed using log rank tests, and MD, enzyme activities, and microscopy data sets were all compared using one- and two-way analyses of variance. \( P \) values less than .05 were considered significant.

RESULTS

Survival and Mortality Rates

The survivorship of the DR flies exceeded that of the Control flies by approximately 50%–60% for both the flies used for enzyme assays (log rank test, \( \chi^2 = 138.43, p = .0000 \)) and EM studies (log rank test, \( \chi^2 = 307.58, p < .0001 \)).

Protein Content

There was no significant effect of age on whole-fly homogenate protein content (Figure 1a; \( F \) ratio = 3.0, \( p = .089 \)). Control flies had significantly higher protein content than did DR flies at all ages (\( F \) ratio = 39.5, \( p < .0001 \)), and even when flies of similar survival stages (Control days 22 and 35 vs DR days 47 and 53) were compared.

Mitochondrial protein per fly (Figure 1b) for DR flies increased significantly with age up to day 35 (\( F \) ratio = 87.4, \( p < .0001 \)) after which it then decreased. Mitochondrial protein content did not differ significantly with age between DR and Control groups (\( F \) ratio = 0.92, \( p = .346 \)). However, when similar survival stages were compared (Control days 22 and 35 vs DR days 47 and 53), Control flies had significantly higher mitochondrial protein content than did DR flies. To summarize, these results (Figure 1) indicate that DR lowers total protein content in flies but does not alter mitochondrial protein content except at very young ages.

Enzyme Activities

The enzyme activities from whole-fly homogenates and mitochondrial fractions were expressed both as activity per fly and activity per mg protein for two main reasons: (1) to provide more information about the metabolic capacity of flies in the DR and Control groups, and (2) to ascertain that any observed differences in MD were not just a reflection of differences in protein content between DR and Control flies.

Whole-fly homogenate activities.—Enzyme activities per mg protein for the four enzymes are shown in Figure 2. The inner membrane enzymes’ (COX and ANT) activities both declined significantly with age (COX: \( F \) ratio = 10.8, \( p = .0016 \); ANT: \( F \) ratio = 48.23, \( p < .0001 \), respectively) for
DR and Control flies with no significant difference between the two groups at any age. However, when similar survival stages were compared (Control days 20 and 29 vs DR days 35 and 43), COX enzyme activities were significantly higher for Control than for DR flies (F ratio = 36.6, p < .0001), but ANT activity remained unaffected by survival stage (F ratio = 0.005, p = .94).

Matrix enzymes (CTS and MnSOD) did not show any uniform pattern: CTS activity peaked at an intermediate age and then declined thereafter, whereas MnSOD activity significantly increased with age for both Control and DR flies (F ratio = 9.6, p < .02). When same age flies were compared, CTS activity was higher for Control than for DR flies on day 20 but much lower on day 29 (p < .0001). However, there was no effect of survival stage on both CTS and MnSOD enzyme activities between Control and DR flies. When expressed in terms of activity per fly, all four enzyme activities mirrored the activity patterns observed earlier (Figure 2), indicating that any differences in enzyme activities between DR and Control flies were independent of the protein concentrations.

Mitochondrial activities.—Mitochondrial activities per mg protein (Figure 3) declined with age for both inner membrane enzymes (COX: [Control F ratio = 73.4, p < .0001 vs DR F ratio = 17.8, p < .0001] and ANT [Control F ratio = 6.1, p < .043 vs DR F ratio = 48.2, p < .0001]). When flies of similar ages were compared, there were no significant differences in COX activity between DR and Control groups at any age. ANT activity was, however, significantly higher for DR than for Control flies of similar age on day 8 (p < .0001). Comparison of both COX and ANT activities at similar survival stages (Control days 20 and 29 vs DR days 35 and 43) showed that neither were affected by survival stage of the flies.

CTS activity per mg protein (Figure 3) was higher for Control than for DR flies on days 8 and 20 (p < .0001), but decreased sharply thereafter and was lower than DR on day 29 (p < .0001). When equivalent survival stages were compared (Figure 3; Control day 20 vs DR day 35 and Control day 29 vs DR day 43) there were no significant differences in enzyme activity per mg protein between the two groups suggesting that DR only delayed the age-dependent decrease in CTS activity. MnSOD activity per mg protein (Figure 3) declined with age for both Control (F ratio = 9.65, p < .017) and DR (F ratio = 43.5, p < .0001) flies; however, Control flies had higher MnSOD activity on days 20 (p < .023) and 29 (p < .021). When similar survival stages were compared (Control days 20 and 29 vs DR days 35 and 43), MnSOD activity was still lower for
DR flies suggesting than DR did not seem to prevent the age-dependent decrease for MnSOD activity.

The mitochondrial activities per mg protein for all four enzymes were also reflected in terms of activities per fly, again indicating that enzyme activities were not influenced by differences in protein content between DR and Control flies. Therefore, to summarize these results, DR appeared to delay the age-dependent decline in COX, ANT, and CTS activities, but did not seem to have any effect on MnSOD activity.

Mitochondrial Protein Density

MD calculated using the inner membrane enzymes (COX and ANT) showed a different picture from that calculated using the matrix enzymes (CTS and MnSOD) (Figure 4). The COX and ANT measurements both showed MD decreasing with age for DR and Control groups (COX: F ratio = 8.17, p < .0058; ANT: F ratio = 12.58, p < .0009), a trend that was identical to that of the enzyme activities (Figure 3). The ANT graph (Figure 4) shows that MD was significantly higher for the Control group on days 8 (p < .01) and 20 (p < .05). However, comparison of MD between DR and Control flies at equivalent survival stages (ANT) shows that the difference was not significant.

The matrix enzymes CTS and MnSOD (Figure 4) showed that MD increased with age (CTS: Control F ratio = 51.41, p < .0002; MnSOD: Control F ratio = 61.57, p < .0001 vs DR F ratio = 122.96, p < .0001). For CTS, MD was higher in the DR group compared to Control on days 8 and 20 (p < .01), but was much lower than DR on day 29 (p < .001). Comparison of equivalent survival stages (MnSOD: Control days 20 and 29 vs DR days 35 and 43) showed that MD was still higher in DR flies, suggesting that the difference in MD between DR and Control flies was not affected by the age of the flies.

MD obtained by the enzyme methods should be independent of method used; therefore, averaging all four panels would give an unbiased and more representative effect of DR on this parameter. The average MD plot (Figure 4) shows an effect of neither age (F ratio = 0.54, p = .47) nor food (F ratio = 0.05, p = .83) on MD in flies. Comparison of equivalent survival stages (Control days 20 and 29 vs DR days 35 and 43) also showed no significant effect of DR on MD.

EM Results

Typical electron micrographs of flight muscle mitochondria from Control and DR flies are shown in Figure 5, a and b, respectively. The mitochondria from DR flies (Figure 5b)
appear larger than those from Control flies (Figure 5a). The myofiber diameters from DR flies also appear larger than those from Controls.

Mitochondrial volume density (Figure 6a) decreased with age in both Control and DR groups ($F_{ratio} = 17.66, df = 2, p < .0001$), and was lower in Control flies ($F_{ratio} = 13.20, df = 1, p = .0013$). When comparisons are made between the DR and Control flies at equivalent survival stages, i.e., days 24 and 28 for Control flies versus days 48 and 53 for DR flies, Control flies had lower mitochondrial volume density.

Figure 4. Mitochondrial protein density calculated from the specific activities of inner membrane (cytochrome c oxidase [COX] and adenine nucleotide translocase [ANT]) and matrix (citrate synthase [CTS] and manganese superoxide dismutase [MnSOD]) enzymes as described in the text. Each point is mean ± standard error of triplicate determinations. *Values significantly different from Control are ANT: day 8 ($p < .01$) and day 20 ($p < .002$); CTS: days 10, 20, and 29 ($p < .01$); MnSOD: days 20 and 29 ($p < .001$).
Figure 5. Typical electron micrographs of flight muscle mitochondria from dietary restriction (DR) and Control flies. a and b, Low magnification images ($\times$3500) of flight muscle showing arrangement of mitochondria within tissue. a is from a Control fly and b is from a DR fly. c-f, High magnification ($\times$35,000) images of flight muscle mitochondria from day 12 individuals; c and f are from Control flies; d and e are from DR flies. a and b are representative of at least six different samples taken from either group of flies on day 12. Double arrows point to the mitochondria, single arrows show glycogen granules, and m = flight muscle myofibers.
Figure 6. The effect of diet and age on stereological parameters of flight muscle mitochondria: (a) mitochondrial volume density ($V_v [mt, c]$), (b) mitochondrial inner membrane surface density ($S_v [mt,mt]$), (c) mitochondrial outer membrane surface density per mitochondrion ($S_v [mt,mt]$), (d) mitochondrial outer membrane surface density per cell ($S_v [mt,c]$), and (e) mitochondrial inner membrane surface density per cell. Data shown are mean ± standard error of the mean for $N = 5–6$ different samples. Open circles represent dietary restriction (DR) animals; closed circles represent Control animals.
DISCUSSION

Dietary restriction (DR) flies showed a slight increase in volume density with survival stage, whereas the Controls showed a sizeable decrease.

Mitochondrial inner membrane surface density (Figure 6b) did not differ significantly between DR and Control flies of comparable age and survival stages (F ratio = 0.2677, df = 2, p = 0.7669). When flies at equivalent survival stages were compared, DR flies had significantly lower inner membrane surface density (F ratio = 6.5827, df = 1, p = 0.0195) compared to Control flies but there was no significant effect of survival stage (Figure 6b: F ratio = 0.7785, df = 1, p = 0.3892).

The mitochondrial outer membrane surface density per mitochondrion (Sv (mt,mt)) (Figure 6c) decreased with age for DR and Control flies (F ratio = 4.0879, df = 2, p = 0.0286) and was much higher for DR than Control on day 12. When Sv (mt,mt) from equivalent survival stages was compared, no significant differences were observed between the two groups (F ratio = 1.9233, df = 1, p = 0.1845).

Mitochondrial outer membrane surface density per cell (Sv (mt,c)) (Figure 6d) was significantly lower in Control than in DR flies of comparable age (F ratio = 38.4411, df = 1, p < 0.0001), and this difference was not affected by age. When Sv (mt,c) was compared between DR and Control flies at comparable survival stages, the effect of DR was not significant (F ratio = 0.8134, df = 1, p = 0.7377), suggesting that DR only affects Sv (mt,c) at younger ages.

Figure 6e shows the mitochondrial inner membrane surface density per cell (Sv (mt,c)) or mitochondrial inner membrane surface area as a ratio of tissue volume for the two groups of flies. Inner membrane surface density was calculated by multiplying the mitochondrial volume density (Figure 6a) by the inner membrane surface density per mitochondrion (Figure 6b). It is a more appropriate measure of mitochondrial density because: (1) it reflects the actual ROS-generating capacity per cell and (2) it gives the most comparable measurement to mg mitochondrial protein per mg whole-homogenate protein (MD) shown in Figure 4. Figure 6e shows that there was no significant effect of age on inner membrane surface density for DR (F ratio = 1.0845, df = 1, p = 0.3742) or Control (F ratio = 0.0155, df = 1, p = 0.9212) groups; neither were there any significant differences between the two groups of flies with age (F ratio = 0.2608, df = 1, p = 0.6313).

In summary, the EM results collectively show that, on average, mitochondrial density did not significantly differ with age between Control and DR flies (Figure 6e), although other parameters such as size (Figure 5) and morphology of mitochondria could be altered with age and show significant differences between DR and Control groups (Figure 6, a–d).

**Discussion**

The overall aim of this study was to test if the effects of DR on life span were mediated through changes in MD. Our working hypothesis was that, if DR lowers ROS production by decreasing the number of ROS-producing organelles, then MD should decrease, rather than increase, in DR flies compared to Controls. The enzyme method showed differences in mitochondrial activities between DR and Control flies (Figures 2 and 3), but there were no associated changes in mitochondrial protein density. The EM method also showed morphological differences between mitochondria from DR and Control flies, but again no differences in inner mitochondrial surface density per cell volume. Taken together, both enzyme and EM results suggest that DR only alters mitochondrial morphology (size, shape, and number) as well as specific enzyme activities, but does not alter mitochondrial content per cell. Support for this suggestion comes from the mitochondrial protein content per fly (Figure 1b), which did not differ significantly between DR and Control flies to the same extent as did total protein content per fly (Figure 1a). Whole-fly homogenate protein content (Figure 1a) was, however, much higher for Control flies indicating that the Control diet also increased protein content of other cellular organelles apart from the mitochondria. Genome-wide profiling of genes associated with aging in *Drosophila* showed that genes involved in cell growth and metabolism were overly expressed in fully fed (Control) compared to DR flies (38), and may possibly be related to the increase in whole-fly homogenate protein content for Control flies from this study.

MD calculated using the enzyme methods should be independent of method used, hence the opposite trends between inner membrane and matrix enzymes (Figure 4) suggest either that: (1) the inner membrane and matrix enzymes were assessing different properties of the mitochondria or (2) there were methodological or technical errors associated with the use of enzymes from the two mitochondrial compartments. The first possibility is highly unlikely because any of the measurements cancel out in the calculation of MD and the final result becomes independent of the enzyme used. However, the second possibility is more likely given the observed trends in the activities of the four enzymes. Fluctuations in CTS activity as well as the opposite trends shown by MnSOD activities from whole-fly homogenates and mitochondrial fractions (Figures 2 and 3) strongly suggest loss of enzyme activity from the matrix possibly due to leakage during isolation of mitochondria. For example, 10%–15% of the CTS activity could be detected in the supernatants of the mitochondrial fractions from 8-day-old flies (result not shown) confirming this supposition. Loss of enzyme from the matrix would give a progressive underestimate of MD using the matrix enzymes (because the mitochondrial preparation has lost matrix contents but not membranes to the same extent), and a corresponding overestimate using the membrane enzymes (because there will be some membranes that lack matrix, so more membranes per mg protein in the mitochondrial fractions).

The EM results may support earlier findings that mitochondrial numbers in vivo decrease with age (48,49); however, mechanisms through which mitochondrial numbers fluctuate in vivo are still obscure. Mitochondrial biogenesis in biological systems is a poorly understood process (50), and processes of mitochondrial fusion (51) and fission (52) that may be involved in regulating mitochondrial numbers in vivo are still poorly understood. In rodents it has been shown that chronic energy deprivation (caloric restriction) induces mitochondrial biogenesis through
Metabolic adaptations that involve both AMP (adenosine 5'-monophosphate) kinase-dependent (53) and -independent (54) pathways. However, it has not been determined whether similar metabolic adaptations do occur in *Drosophila* under DR. The finding from this study that DR alters mitochondrial morphology (Figure 6, a–d) without altering mitochondrial protein density (Figure 4) or inner membrane density per cell volume in *Drosophila* (Figure 6e) suggests that DR may influence mitochondrial fusion and/or fission events in vivo, but this has yet to be demonstrated.

Mitochondrial populations in tissues are not homogeneous and can be separated into heavy, medium, or light fractions according to their degree of maturation and size (55). It has been shown for brown adipose tissue from rats that chronic interventions such as overfeeding or prolonged fasting produce changes mainly detected in the heavy mature mitochondrial populations, whereas acute short-term fasting or cold exposure produces adaptive changes in light immature mitochondrial fractions (56,57). Apparently lifelong (chronic) DR provides a different physiological stimulus from full feeding and may alter mitochondrial populations differently between DR and Control animals. Hence the assumption that the cell population from which mitochondria are harvested is the same in both DR and Control flies may be incorrect, and determinations of MD using the enzyme method may always need confirmation by at least one other method, as we did here using EM.

**Summary**

DR did not significantly decrease mitochondrial density (mitochondrial protein density or mitochondrial inner membrane surface density per cell) in *Drosophila*. DR, however, appeared to delay the age-dependent decreases in some mitochondrial enzyme activities and morphological changes seen in the Control group of flies. We therefore conclude that life-span extension by DR in *Drosophila* may not be mediated through a reduction in mitochondrial density and, if DR in *Drosophila* extends life span by reducing ROS production, then it does so through mechanisms that operate only in vivo.

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