




Dietary management and physical exercise can improve climbing defects and mitochondrial activity in *Drosophila melanogaster parkin* null mutants

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ABSTRACT

Physical exercise can improve gait, balance, tremor, flexibility, grip strength and motor coordination in Parkinson's disease (PD) patients. Several lines of evidence have also shown the therapeutic potential of dietary management and supplementation in halting the progression of PD. However, there is a lack of research on the combined effects of physical activity and nutrition in the progression of PD. We test the effects exercise and dietary modification in a *Drosophila* model of PD. In this study, we fed *Drosophila parkin* mutants high protein and high carbohydrate diets without and with stearic acid (4 treatments in total). In parallel, we subjected mutants to a regimen of exercise using a purpose-built 'Power tower' exercise machine. We then measured climbing ability, aconitase activity, and basal mitochondrial ROS levels. We observed that exercising *parkin* mutants fed the high protein diet improved their climbing ability and increased aconitase activity. There was an additional improvement in climbing and aconitase activity in exercised *parkin* mutants fed the high protein diet supplemented with stearic acid. No benefits of exercise were seen in *parkin* mutants fed the high carbohydrate diet. Combined, these results suggest that dietary management along with physical activity has potential to improve mitochondrial biogenesis and delay the progression of PD in *Drosophila parkin* mutants.

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Introduction


The primary goal of this study is to investigate whether dietary management with physical exercise, is more effective than either alone, in delaying the onset of Parkinson's disease (PD) like symptoms in a *Drosophila* model of PD. Most generally, there are at least four approaches that can delay the onset of PD and improve health span. We define health span as the length of time that a person is healthy – not just alive. First, overall well-being may be improved by a general improvement in health and enhanced immune function. Second, improving the strength and vigor of individuals with any neurological disease is expected to slow the development of PD. Third, strategies targeted towards the molecular mechanisms underpinning PD are a highly specific approach to improve the health of people with the disease. Fourth, interactions between both environmental and genetic variables may provide novel treatment opportunities.

A general improvement of diet is expected to increase well-being and immune function. Seidl

et al. [1] outlined the foods that are linked to a reducing risk or progression of PD in humans. These include fruits and vegetables, and what has been referred to as a 'Mediterranean diet,' although this relationship is not straightforward since pomegranate juice exacerbated oxidative stress in a rotenone model of PD [2]. A high carbohydrate diet has been shown to increase longevity in mice and flies, and in a fly model of PD, it delays the onset of PD like symptoms [3–5]. Here, we follow our previous study and include 1:2 Protein: Carbohydrate (P:C) and 1:16 P:C diets [5]. Protein and carbohydrate are the two-major energy-yielding macronutrients in the fly diet and these ratio's span range naturally fed upon by flies in nature.

Developing novel methods of stimulating neuroplasticity is a promising treatment approach to counterbalance maladaptive influences and alleviate symptomologies. One non-pharmacological approach with significant and direct impacts on neuroplasticity is aerobic exercise [6], however, there is debate about the benefit of aerobic exercise training as a specific

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treatment for PD [7–9]. Here, *Drosophila* flies fed four diets were exercised by employing their negative geotaxis behavior [10]. Prior to this approach, experiments using insect models to examine the relationship of exercise on aging and disease utilized the continuous increase in flying activity as an exercise stimulus. Under these highly challenging conditions, exercise was shown to increase oxidative damage and to decrease lifespan [11,12].

Targeted strategies are the most specific approach to improve the health of people with PD. In humans, delaying the onset and severity of motor symptoms is beneficial for patients with PD as it can delay the onset of levodopa therapy and the subsequent onset of motor complications [13]. In this study, we add stearic acid to the high protein and high carbohydrate diets, conduct an exercise program over 11 days, and then assess aspects of physiology and mitochondrial health. We included stearic acid because it has been shown to act as a signaling molecule to improve survival and mitochondrial function in *Drosophila parkin* mutants [14].

Here, we test male *Drosophila parkin* mutant flies as a model for PD. *Drosophila parkin* mutants show a phenotype that highly resembles PD, including diminished climbing and flight ability, reduced mitochondrial functions, reduced longevity, and muscle degeneration [15,16]. *Drosophila parkin* is the homolog of human *PARK2* and mutation of the *PARK2* gene is a major cause of autosomal recessive juvenile PD [17,18].

Climbing ability, mitochondrial aconitase activity and basal mitochondrial reactive oxygen species (ROS) were quantified as measures of the nutritional and exercise regimes. We have previously shown improvement in the climbing ability of *parkin*-males fed 1:16 P:C food as compared to a 1:2 P:C diet [5]. Mitochondrial aconitase is an enzyme in the tricarboxylic acid (TCA) cycle, and the catalytic loss of activity is an indicator of oxidative damage [19]. Elevated levels of ROS produced by mitochondria as a by-product of energy production causes oxidative stress [20], which is important in the progression of PD [reviewed in, [21]].

The overall goal of the research is to determine an optimum set of modifications that delays the onset of a Parkinsonism like phenotype in *parkin*-males. Here, we hypothesized that moderate exercise and

the dietary addition of stearic acid would improve physiological and mitochondrial functions in *parkin*-males. We have previously shown that *parkin* mutants have a longer lifespan when fed the 1:16 P:C compared to those fed the 1:2 P:C diet. *Parkin* mutants fed the high carbohydrate diet also have delayed climbing deficit, increased resistance to starvation, and improved mitochondrial functions [5]. We had also shown that *parkin*-flies had significant improvement in all physiological and mitochondrial functions assayed when stearic acid was added to high protein 1:2 P:C but not high carbohydrate 1:16 diet [22].

Results

Exercise training with climbing

To test our purpose-built Power tower, *yw* flies were exercised following the ramping protocol of Piazza and colleagues [10] and climbing assays conducted every second day from 3 d to 28 d of age. Relative to unexercised flies, the climbing ability of exercised flies declined more slowly, and there was a clear difference in this physiological trait until the cessation of exercising at 24 d of age (Figure 1). Student's t-test showed a significant difference between the slopes of exercised and unexercised flies during the exercise period ($t_{116} = 2.25$, $P = 0.026$).

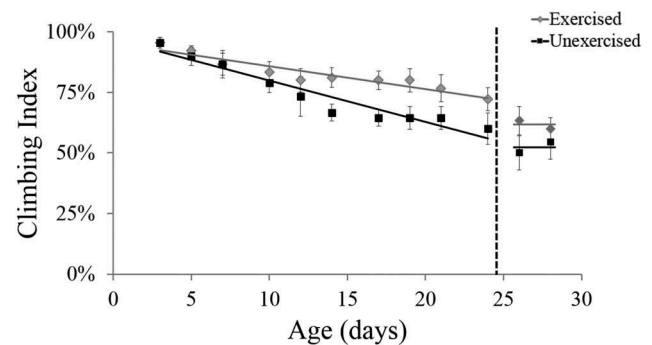


Figure 1. Climbing index of *yw* males. Flies were exercised from day 3 until day 24, five days a week. The left side of the dashed line is the exercise period, and right side of the dashed line is the post-exercise period. A total of 90 exercised and 90 unexercised *yw* flies were used. Each treatment had 6 replicates with 15 flies per replicate. During week one they were exercised for 2 hours/session, during week two for 2.5 hours/session and during week three for 3 hours/session. The lines indicate the regression for exercise and unexercised flies (for exercised flies $y = -0.0094x + 0.95$ and for unexercised flies $y = -0.0172x + 0.97$). Symbols indicate mean, and bars indicate s.e.m.

Following the cessation of exercise, its benefits were quickly lost (Figure 1). These data are similar to that reported by Piazza and colleagues [10], though we observed a slight extension in the benefit of exercise.

The *parkin*- males are known to have motor disorders [16], so we optimized the exercise program for 11 d old flies fed 1:2 P:C ratio diet. The most noticeable improvement came from Regimen 4 where *parkin*- males were subjected to 30 mins of exercise every alternate day (Figure 2). ANOVA showed a significant effect of regimen ($F_{5,30} = 8.11$, $P < 0.001$). Dunnett's multiple comparison tests demonstrated significantly low climbing index of regimen 3 ($Q = 2.65$, $P = 0.015$) and an improved climbing index on regimen 4 ($Q = 2.65$, $P = 0.002$) compared to the unexercised *parkin*-.

To further test whether the exercise regimen influenced the number of inactive flies we recorded the flies that climbed less than 20 mm. Regimen 4 had lowest numbers of inactive flies while regimen 3 had the highest (Supplementary Figure 2). ANOVA showed a significant effect of regimen ($F_{5,30} = 5.57$, $P < 0.001$) and Dunnett's multiple comparison test showed significantly higher inactive flies on regimen 3 ($Q = 2.65$, $P = 0.0008$). As a result of these climbing results, Regimen 4 was

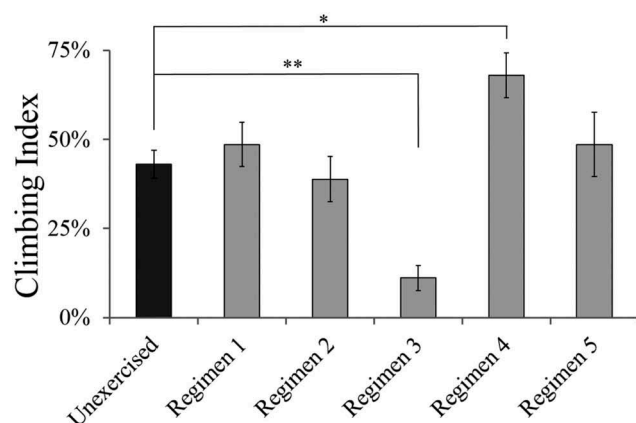


Figure 2. Optimization of exercise regimen for *parkin*- males using the climbing assay. *Parkin*- males had highest climbing index when exercised for 30 mins every alternate day (Regimen 4). Regimens 1, 2 and 3 exercised *parkin*- for 30, 60 and 120 mins every day, respectively. Regimens 4 and 5 exercised *parkin*- males for 30 and 60 mins, respectively on alternate days. Each regimen had 6 replicates of 12 *parkin*- males for 72 *parkin*- males/regimen (a total of 432 flies were included in the study). The flies were fed 1:2 P:C ratio diet throughout the exercise period. Bars indicate means and error bars indicate standard error. Significance determined by Dunnett's test at * $P < 0.05$, ** $P < 0.01$ (see text for details).

followed for the remainder of the assays. A limitation of this optimization protocol was that we did not test flies fed the 1:16 P:C food.

Climbing ability with exercise and dietary modification

PD is characterized by a battery of physiological symptoms including slowness of movement [23]. We measured the climbing ability of unexercised and exercised *parkin*- flies fed four isocaloric diets. Both exercise and stearic acid improved the climbing ability of *parkin*- flies fed 1:2 P:C ratio diet but not the 1:16 P:C diet (Figure 3A). For *parkin*- fed 1:2 P:C diet, ANOVA showed significant main effects of stearic acid supplementation and exercise ($F_{1,28} = 23.9$, $P < 0.001$; $F_{1,28} = 8.02$, $P = 0.01$, respectively) with no significant interaction ($F_{1,28} = 0.30$, $P = 0.58$). For the 1:2 P:C diet, Student's t-tests showed a significant effect of stearic acid ($t_{28} = 4.88$, $P < 0.0001$) and exercise ($t_{14} = 3.44$, $P = 0.003$). For *parkin*- flies fed the 1:16 P:C diet, ANOVA reported no significant effects of stearic acid supplementation, exercise or their interaction ($F_{1,28} = 0.12$, $P = 0.73$; $F_{1,28} = 1.4$, $P = 0.25$ and $F_{1,28} = 1.65$, $P = 0.21$, respectively).

Aconitase activity with exercise and dietary modification

Aconitase activity is an indicator of mitochondrial efficiency and loss of activity in samples treated with pro-oxidants has been interpreted as a measure of oxidative damage [19]. Exercise tended to increase aconitase activity of *parkin*- males fed both diets but the effect was more marked in flies fed the high protein 1:2 P:C diet (Figure 3B). For flies fed 1:2 P:C diet, ANOVA demonstrated a significant effect of exercise ($F_{1,20} = 6.62$, $P = 0.02$) but no significant effect of stearic acid or exercise by stearic acid interaction ($F_{1,20} = 2.75$, $P = 0.11$ and $F_{1,20} = 0.20$, $P = 0.65$, respectively). Student's t-test showed a significant difference of exercise in *parkin*- fed 1:2 diet without stearic acid ($t_{10} = 2.44$, $P = 0.034$). For flies fed the 1:16 P:C diet, there was no significant effect of exercise, stearic acid supplementation, or their interaction ($F_{1,20} = 1.40$, $P = 0.25$; $F_{1,20} = 0.12$, $P = 0.73$ and $F_{1,20} = 0.67$, $P = 0.74$, respectively).

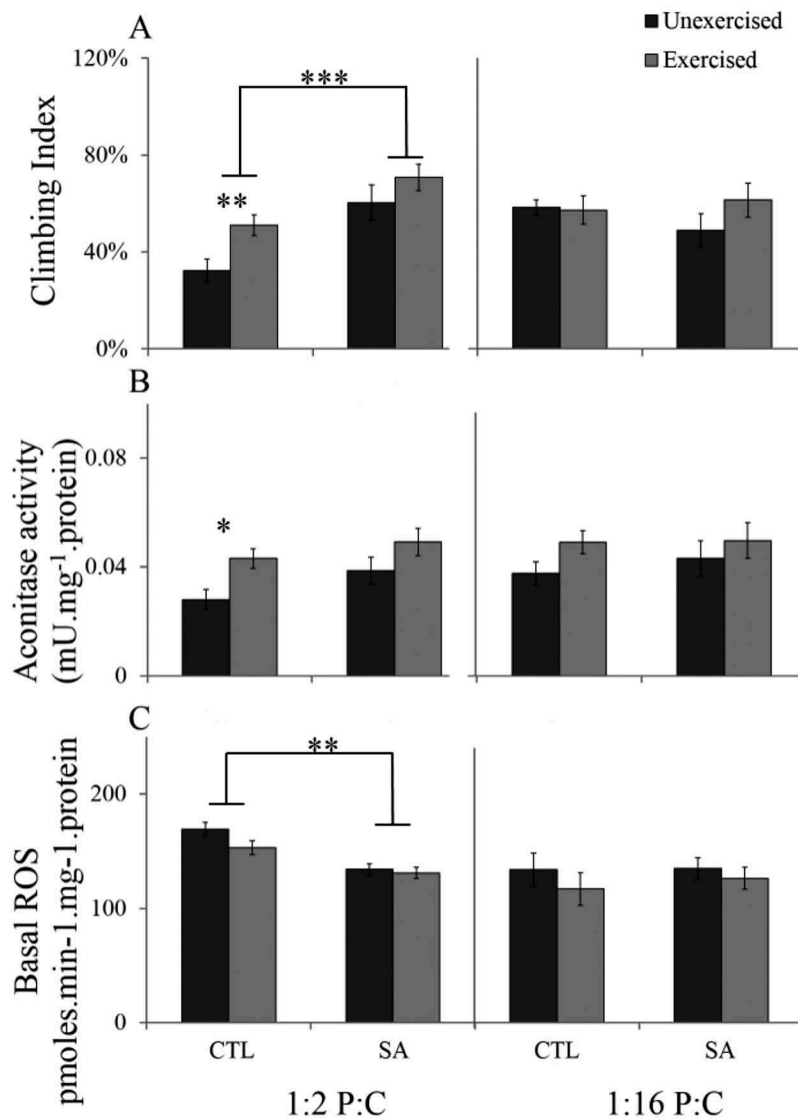


Figure 3. Climbing index, aconitase activity and *Basal ROS* of unexercised and exercised *parkin*- males fed high Protein (1:2 P:C) and high Carbohydrate (1:16 P:C) ratio diet without (CTL) or with stearic acid (SA). A. Climbing. B. Mitochondrial aconitase activity. C Basal ROS. Bars indicate means and bars indicate s.e.m. Significance determined by Student's t-test at *P < 0.05, **P < 0.01 and ***P < 0.001 (see text for details).

Basal mitochondrial ROS with exercise and dietary modification

Basal mitochondrial ROS gives the levels produced at the resting state and are an indicator of mitochondrial coupling efficiency in respiration. Stearic acid reduced basal ROS levels in flies fed the 1:2 P:C diet (Figure 3C). For *parkin*-males fed 1:2 P:C ratio diet, ANOVA demonstrated a significant effect of stearic acid supplementation ($F_{1,20} = 9.04$, $P = 0.01$) and no significant effect of exercise or stearic acid by exercise interaction ($F_{1,20} = 1.04$, $P = 0.32$ and $F_{1,20} = 0.48$, $P = 0.49$, respectively). A Student's

t-test showed a significant effect of stearic acid in *parkin*- fed 1:2 diet ($t_{20} = 3.00$, $P = 0.007$). For *parkin*- fed 1:16 P:C diet, ANOVA showed no significant effects of exercise, stearic acid supplementation or their interaction ($F_{1,20} = 0.11$, $P = 0.74$; $F_{1,20} = 0.72$, $P = 0.40$ and $F_{1,20} = 0.07$, $P = 0.79$, respectively).

Discussion

In humans, exercise and nutritional management are non-invasive strategies to counteract neurological and cognitive disorders [24]. In this study, we

test the influence of dietary intervention and exercise in delaying PD like phenotypes in *parkin* mutant flies. We include high protein and high carbohydrate diets and examine the impact of adding stearic acid and of exercise on climbing ability, aconitase activity, and basal mitochondrial ROS levels. We find that both stearic acid and physical activity improved aspects of physiological health and mitochondrial functions when flies were fed the high protein diet but not the high carbohydrate diet. Collectively, these results suggest that improving mitochondrial function may be one avenue to delay the progression of PD.

Addition of stearic acid to the high protein diet improved climbing ability and basal ROS levels, likely by decreasing mitochondrial proton and electron leak [25–28]. Stearic acid is known to be converted to oleic acid [25], which is incorporated into mitochondrial phospholipids in the rat [26]. Phospholipids make up the characteristic outer and inner membranes that give mitochondria their shape [27]. It is the phospholipids that also give rise to other characteristic mitochondrial structures such as cristae (formed from the invaginations of the inner mitochondrial membrane), the matrix (area within cristae) and the intermembrane space that separates the outer mitochondrial membrane and inner mitochondrial membrane [27]. The incorporation of oleic acid derived stearic acid likely results in increased mitochondrial membrane potential in *Drosophila* [28], which is a critical component of the proton motive force required for ATP. More recently it has also been shown that stearic acid stearylates TFR1, thereby inhibiting its activation of JNK signaling. This leads to reduced ubiquitination of mitofusin, which promotes mitochondrial health through fusion [14].

Moderate exercise improved climbing ability and aconitase levels. Combined, this suggests a general improvement in mitochondrial functions, which may then stimulate antioxidants and decrease oxidative stress. To exercise the flies, we constructed a Power tower [10] and conducted an initial study to develop an optimal exercise program for *parkin* mutant flies. Human and animal studies have reported that regular physical activity can help recovery from brain injury and improve learning and memory in age-related neurodegenerative disorders [29–35]. For example, treadmill exercise is a

promising non-pharmacological approach for reducing the risk of PD and has proven to reduce depression and produce neuroprotective effects in a mouse model of PD [31,34,36]. Aerobic exercise has shown to improve gait, mood, cognition, balance, tremor, flexibility, grip and motor coordination in PD patients [8,9]. More generally, physical activity can be neuroprotective to aging individuals and may help the patients with neurodegenerative disease [29,30,32,37,38]. In a rat model of PD, treadmill exercise improved gait speed and balance, reduced oxidative stress, improved mitochondrial fusion and fission, increased mitochondrial amounts, and potentially attenuated dopaminergic neuron degeneration [39].

We show that exercise improved climbing ability and increased aconitase activity in *parkin*-fed a high protein diet. Further, the addition of stearic acid to the high protein diet enhanced climbing ability. Neither dietary modification nor physical activity significantly improved physiological or mitochondrial functions in flies fed the high carbohydrate diet. One possible explanation for these results is that both nutritional modifications and exercise work, at least partially, by improving mitochondrial health. Mitochondrial dysfunction is emerging as a common feature of PD suggesting pathogenic overlap among the familial, environmental and sporadic disease pathways [40]. Future studies may add rapamycin in the diet to explore the potential for exercise to reduce TORC1 signaling and improve the climbing activity of *parkin* mutants. Rapamycin has shown to increase the survival, aconitase and antioxidant activity in *Drosophila* models [41]. Likely, however, the specific mechanisms will differ due to the complex and dynamic nature of the organelle and its interactions with its cellular environment [42].

Materials and methods

Drosophila strains and husbandry

Initial testing of the Power tower made by us was conducted on *yw* males. Experimentally, *yw; park25/TM6B* were maintained, and *park25/park25 (parkin-)* males were used in the study [22].

Flies were maintained in Carolina 424 *Drosophila* Instant Medium in an incubator set to $23 \pm 1^\circ\text{C}$,

60 ± 5% relative humidity with a 12 h: 12 h light: dark cycle. The densities of flies in bottles were monitored to avoid larval competition. The 1 d old male flies were sorted on the ice and placed into a vial with one diet. Flies were transferred to new vials every 2–3 d to avoid fungal contamination.

Dietary modification

For the initial study with *yw* flies, males were fed on Instant *Drosophila* food (Carolina, Biological Supply). Instant food was used for this study as it is commercially available in multiple countries. For the initial optimizing experiment with *parkin*- males the 1:2 P:C diet was chosen. This diet was selected because the *parkin*- flies had the most diminished climbing ability when fed this diet [5].

For the experimental study with *parkin*- males four isocaloric diets were constructed [22]. Diets have high protein (1:2 P:C) and high carbohydrate (1:16 P:C) and presence/absence of stearic acid. The diets without stearic acid are the controls for those with stearic acid. We calculated and tested a range of stearic acid concentrations and optimized the dietary stearic acid level to 4% suitable for *parkin*- males [22]: a previous study added 10% stearic acid into the fly diet [12]. The amount of yeast (MP Biomedicals, catalog no. 103304), sucrose (MP Biomedicals) and stearic acid (Sigma Aldrich) is shown in (Supplementary Table 1). The total calorie content of the diets is 0.65 ± 0.04 kcal.ml⁻¹. The mixture of yeast, sucrose, stearic acid and 1% agar was cooked in the microwave for 5 min per 100 ml of deionized water. After heating the mixture of food, 1 ml of 0.01% phosphoric acid, 0.1% propionic acid and 0.1% of nipagen was added as antifungals agents, 2 ml of food was then transferred into 30 ml vials. The food was prepared each week and stored at 4°C until used.

Exercise training

There is a need to explore the benefits and limitations of exercise in PD [7]. Here, the Power tower was constructed following Piazza and colleagues [10] (Supplementary Figure 1). The instrument consists of a motor attached to a rotating arm. As the arm rotates along the lever, it raises the platform. At the top, the platform is released. Rubber shock absorbers on the base of the platform reduced excessive shock and the flies are knocked to the base of the vial. As the platform is raised the flies walk upwards using their geotaxis behavioral response.

To confirm that the machine we built performed like that reported by Piazza and colleagues [10] we conducted an initial study following their protocol. We collected 1 d old *yw* flies and kept in them in groups of 15. Flies were exercised five days a week, utilizing a ramping schedule. In the first week, flies were exercised for 2 hours/session, week two for 2.5 hours/session, and week three for three hours/session. For exercising, flies were first transferred to a fresh vial containing 2 ml of 1% agar and then covered with sponge stopper before the exercise. The vials were inverted so that during the exercise they landed on a soft surface. For, non-exercised flies, the sponge stopper was inserted deep into the vial so that flies received the same stress but had reduced capacity for geotaxis induced exercise. No exercise was performed after 24 d which was termed the post-exercise period.

To optimize the exercise program for *parkin*- flies, 1 d old flies were carefully sorted on ice, fed the 1:2 P:C ratio diet (without stearic acid) and climbing assay conducted at 11 d of age. We selected the 1:2 P:C diet because our previous study showed that *parkin*- fed 1:2 P:C ratio had significant climbing defects [5]. The 11 d age was selected because *yw* flies started to show

Table 1. Exercise regimens for *parkin*- males. The time of exercise ranges from 30 to 120 min per day. Exercising was done every day and every alternate day. Climbing Index was performed 3 h after the exercise on 11 d. A total of 72 flies were assayed for each regimen.

Age	1	2	3	4	5	6	7	8	9	10	11
Regimen 1	30	30	30	30	30	30	30	30	30	30	30
Regimen 2	60	60	60	60	60	60	60	60	60	60	60
Regimen 3	120	120	120	120	120	120	120	120	120	120	120
Regimen 4	30	Rest	30	Rest	30	Rest	30	Rest	30	Rest	30
Regimen 5	60	Rest	60	Rest	60	Rest	60	Rest	60	Rest	60

exercise-induced differences at this age and a previous study including these diets, but not including exercise, measured climbing ability of *parkin*- males at this age [22].

To begin the exercise regimen, each vial included 12 *parkin*- males. The different exercise regimens are shown in Table 1. During the exercise period, no deaths were recorded in unexercised flies or regimens 1 and 4. However, 13 flies died in regimen 2, 19 in regimen 3 and 7 in regimen 5. At 11d of age, replacement exercised flies from the appropriate regimen were added to replace dead flies so that there was 12 *parkin*-males/vial. We employed this approach so that the 11 d climbing assays were not biased by small numbers. A preliminary study showed that there were no differences in food consumption between exercised and unexercised 11 d *parkin*-males ($F_{1,49} = 0.03$, $P = 0.85$).

Climbing ability

We tested the climbing ability of *yw* and *parkin*-males following Le Bourg and Lints [43] with slight modification. For each diet, flies were raised in groups of 12 per vial. A horizontal line was drawn 80 mm above the bottom of the 110 by 27 mm vial, and another same vial was used as a cover to provide additional space. We also recorded flies that were inactive (between 0–20 mm). The flies were acclimated for 10 min at room temperature. The flies were given 20 s to climb the vial, and the percentage of flies that crossed each mark each time was recorded.

A total of 90 exercised and 90 unexercised *yw* males were tested and 360 exercised, and 72 unexercised *parkin*- flies were assayed. Following each climbing assay, the *yw* males were returned to their vials to continue their exercise regime. For *yw* flies, the climbing assay was carried out on 3, 5, 7, 10, 12, 14, 17, 19, 21, 24, 26 and 28th day. For the *parkin*- flies, the climbing assay was conducted only at 11 d of age. After this assay flies were processed for aconitase activity and basal ROS.

Mitochondrial aconitase activity

Exercised wild-type flies have higher aconitase activity than unexercised ones [10], but the influence of

exercise on *parkin*- males is unknown. Aconitase activity of *parkin*- flies was measured following the conversion of citrate into α -oxoglutarate coupled with the reduction of NADP, as previously described by [44]. We extracted mitochondria from thorax muscles following Melvin and Ballard [45]. The mitochondrial pellets were subject to four freeze-thaw cycles in liquid nitrogen in buffer containing 154 mM Tris HCl, pH 7.4, and 5 mM citrate. The reaction mixture contained 27 mM Tris-HCl, pH 7.4, 5 mM sodium citrate, 0.2 mM NADP, 0.6 mM $MnCl_2$ and 1 unit of isocitrate dehydrogenase (where one unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 μ mol of isocitrate from citrate per min at pH 7.4 at 30°C). The slope of NADPH formation over time indicated the activity of aconitase. Absorbance at a wavelength of 340 nm using a SpectraMax Plus spectrophotometer and SoftMax Pro software was followed over time at 30°C at (Molecular Devices Corp., Sunnyvale, CA, USA). A total of 48 mitochondrial extractions were assayed.

Basal mitochondrial ROS

We have previously shown basal mitochondrial ROS is higher in *parkin*- males fed the 1:2 P:C ratio diet than the 1:16 P:C food [5], but the influence of exercise is not known. ROS was assayed from mitochondria isolated from thorax muscles of flies raised in groups of 10. Mitochondria were extracted, and basal ROS was determined using Amplex Red (ThermoFisher Scientific) as previously described [45,46]. Basal ROS levels were determined by quantifying levels before the addition of ADP. H_2O_2 reacts with Amplex Red in the presence of horseradish peroxidase to form the oxidative product resofurin, which has a maximum absorbance at 560 nm [47]. No substrate controls showed no treatment effects [48]. Results were recorded every 1 min for 15 min using the SpectraMax Plus spectrophotometer and SoftMax Pro software (Molecular Devices Corp., Sunnyvale, CA, USA). The amount of ROS production in each well was expressed as pmol of H_2O_2 production per minute per mg of protein. A total of 48 mitochondrial extractions were assayed from four experimental diets in 6 replicates.

Statistics

Student's t-test on slopes tested whether our Power tower replicated the results of Piazza and colleagues [10]. Analysis of variance (ANOVA) with JMP® v12 (2007 SAS Institute, Cary, NC, USA) followed by Dunnett's test was used to determine the significance in different exercise regimen tests. The high protein and high carbohydrate dietary studies were analyzed separately because visual inspection of the data suggested interesting high protein diet-specific effects. Climbing index data were arcsine square root transformed. ANOVA followed by Student's t-tests were conducted to analyze the climbing, aconitase and basal ROS studies with *parkin*-males where diet and exercise were manipulated.

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Disclosure statement

No potential conflict of interest was reported by the authors.

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