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Mito-nuclear interactions modify *Drosophila* exercise performance

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Abstract

Endurance exercise has received increasing attention as a broadly preventative measure against age-related disease and dysfunction. Improvement of mitochondrial quality by enhancement of mitochondrial turnover is thought to be among the important molecular mechanisms underpinning the benefits of exercise. Interactions between the mitochondrial and nuclear genomes are important components of the genetic basis for variation in longevity, fitness and the incidence of disease. Here, we examine the effects of replacing the mitochondrial genome (mtDNA) of several *Drosophila* strains with mtDNA from other strains, or from closely related species, on exercise performance. We find that mitochondria from flies selected for longevity increase the performance of flies from a parental strain. We also find evidence that mitochondria from other strains or species alter exercise performance, with examples of both beneficial and deleterious effects. These findings suggest that both the mitochondrial and nuclear genomes, as well as interactions between the two, contribute significantly to exercise capacity.

Keywords

Drosophila; exercise; mitochondrial; nuclear; interaction

1. Introduction

Endurance exercise is increasingly recognized as an intervention that profoundly reduces the incidence of multiple important age-related diseases, including cancer, diabetes, and cognitive decline (Cassilhas et al., 2016; Thomas et al., 2017; Zanuso et al., 2017). Despite the pervasive benefits of exercise, the molecular mechanisms driving these effects are only just beginning to be understood. One important mechanism mediating the effects of endurance exercise is thought to be maintenance of mitochondrial integrity and quality (Bo et al., 2010; Kang et al., 2013; Laker et al., 2014b).

Mitochondrial dysfunction increases with age in humans (Dai et al., 2012) and model organisms (Kang et al., 2013; Owusu-Ansah et al., 2013), leading to reduced respiratory function, and increased accumulation of reactive oxygen species (Chan et al., 2010). These deficits have been associated with increased incidence of cardiovascular (Liang and Kobayashi, 2015) and neurodegenerative (Moran et al., 2012) diseases, as well as general metabolic dysfunction (Ziegler et al., 2015).

Endurance training has long been known to stimulate mitochondrial biogenesis (Irrcher et al., 2003). More recently, it has become clear that training also improves mitochondrial quality (Yan et al., 2012), and this improvement is dependent on induction of mitophagy (Venditti et al., 2013). This mechanism appears to be broadly conserved, as it has been observed in both vertebrate (Booth et al., 2015) and invertebrate (Laker et al., 2014b) models.

Effective mitochondrial activity depends on cooperative function between proteins encoded by the nuclear and mitochondrial genomes (Rand et al., 2004; Tranah, 2011). Coordination between the products of these genomes is essential for proper function under normal conditions, or during stressful conditions such as endurance exercise (Ryan and Hoogenraad, 2007). While endurance exercise has been observed to induce substantial changes to chronic expression of nuclear genes (Coffey and Hawley, 2007; Sujkowski et al., 2015), less is known about the coordination of these changes with the mitochondrial genome.

Substantial individual variation in the response to identical endurance exercise paradigms exists within the human population (Bouchard et al., 2012; Puthucheary et al., 2011) and between strains of model organisms (Britton and Koch, 2001; Mendez et al., 2016). With increasing interest in personalized genomic approaches to medicine, understanding the genetic bases of this individual variation is an important goal. One important source of this variation could be interactions between the mitochondrial and nuclear genomes. Here, we seek to gain greater understanding of the contributions of the mitochondrial and nuclear genomes to exercise adaptation using unique populations of “mito-switch” *Drosophila*. These lines harbor mitochondria from exogenous fly lines of three types: 1) a line selected for greater longevity over many generations, 2) different strains of *Drosophila melanogaster*, 3) other species from the *Drosophila* genus.

Using a negative geotaxis-based paradigm for endurance exercise (Piazza et al., 2009a; Tinkerhess et al., 2012a), we assessed the baseline speed, endurance, flight and cardiac performance of wild-type *Drosophila*. We then compared them to flies with an identical nuclear genome, but different mitochondrial genomes (hereafter mitotypes or mtDNAs). We further compared the ability of each combination of mitotype/nucleotype to adapt to three weeks of chronic endurance exercise.

We find that mitochondria derived from longevity-selected flies were able to confer substantial performance improvements on their original parental line. Mitochondria from exogenous strains or from other *Drosophila* species had complex and variable effects, with both mitotype and nucleotype having significant effects on most assays. These results are

consistent with the ideas that both the mitochondrial and nuclear genomes, as well as interactions between the two, play important roles in determining exercise capacity.

2. Materials and Methods

2.1 Drosophila stocks and maintenance

w^{1118} and *OregonR* were obtained from the Bloomington *Drosophila* stock Center (BDSC). *Ra, La, RaLa*$_{(m)}$, and *LaRa*$_{(m)}$ were described in (Arking, 1987; Soh et al., 2007b). *OreR*$_{(m)}$;*OreR, siI*$_{(m)}$;*OreR, sm21*$_{(m)}$;*OreR, Zim53*$_{(m)}$;*OreR,* $w^{1118}{}_{(m)}$;w^{1118} , *siI*$_{(m)}$;w^{1118} , *sm21*$_{(m)}$;w^{1118}, *OreR*$_{(m)}$;w^{1118}, *Zim53*$_{(m)}$;w^{1118}, and *Zim53*$_{(m)}$;*Zim53*, hereafter referred to as the 'mitoswitch' lines, were described in (Zhu et al., 2014). Note that the RaLa$_{(m)}$ and LaRa$_{(m)}$ stocks list the nuclear genomes first and the mtDNA (m) second, whereas the 'mitoswitch lines list the mtDNA (m) first and the nuclear genome second, separated by a semicolon (e.g., *OreR*$_{(m)}$;w^{1118}). Flies were cultured and housed on standard 10% sucrose 10% yeast medium at 25°C, 50% humidity under 12 hour light/dark cycle. All stocks were confirmed by PCR to be Wolbachia-free at the time of measurement.

2.2 Exercise training

Exercise training was performed as in Piazza et al. (2009a). Briefly, cohorts of at least 880 male flies were collected under light CO_2 anesthesia within 2 hours of eclosion and separated into vials of 20. Flies were then further divided into 2 cohorts of at least 440 flies designated "exercised" or "unexercised". Every morning prior to training, both exercised and unexercised cohorts were flipped onto fresh vials of 10% sucrose, 10% yeast food. Unexercised flies were treated identically to exercised siblings, but had a foam stopper placed low in the vial during exercise training to prevent running while on the exercise apparatus. The exercise device drops the fly vials every 15 seconds in order to repetitively induce negative geotaxis. Exercised flies are free to run to the top of the vial. A program of gradually increasing daily exercise generates significant improvements in mobility (Damschroder et al., 2018).

2.3 Climbing Speed

Each day prior to exercise training, flies were assessed for climbing performance using a rapid iterative negative geotaxis (RING) assay as in Gargano et al. (2005). Flies were transferred to individual polypropylene vials in a RING apparatus and allowed to equilibrate for 1 minute. Negative geotaxis was elicited by sharply rapping the RING apparatus four times in rapid succession. The positions of the flies were captured in digital images taken 2s after eliciting the behavior. Images were analyzed using ImageJ (Bethesda, MD). The relative distance climbed by each fly was converted into quadrants using Microsoft Excel. The performance of 20 flies was calculated as the average of four consecutive trials to generate a single datum. Flies were tested longitudinally 5 times per week for 3–5 weeks to assess decline in negative geotaxis speed with age. Data were further consolidated into pre- and post-training performance normalized to the starting climbing index of each individual cohort. Summary histograms are presented as the average climbing speed of a single cohort during week 1, and after 3 weeks of endurance training. Between assessments, flies were

returned to food vials and housed until the following RING test. Statistical tests and modeling are described in *Statistical Analyses*.

2.4 Endurance

Climbing endurance was measured using the fatigue assay described previously (Damschroder et al., 2018; Tinkerhess et al., 2012a). At least eight vials of 20 flies from each cohort were subjected to the fatigue assay at two time points. Before exercise, flies are tested once on day 5 of adulthood. The cohort is then split into exercised and unexercised groups and tested again on day 25 of adulthood. For each assessment, the flies were placed on the Power Tower exercise machine and made to climb until they were fatigued, or no longer responded to the negative geotaxis stimulus. Monitored continuously, a vial of flies was visually determined to be "fatigued" when five or fewer flies could climb higher than 1 cm after three consecutive drops. A minimum of 8 vials containing 20 flies each was used for each fatigue assessment with each vial plotted as a single datum. Summary histograms are presented as the average runspan of a single cohort during week 1, and after 3 weeks of endurance training. Each experiment was performed in duplicate or triplicate, and runspans were scored blindly when possible. The time from the start of the assay to the time of fatigue was recorded for each vial, and the data analyzed using log-rank analysis in GraphPad Prism (San Diego, CA, USA). In addition, twoway ANOVA was performed in R (R, 2016) comparing genotype x training and nucleotype x mitotype for exercised and unexercised cohorts for all orthogonal experimental groups. Additional log-rank analyses were performed in Prism. Tables and graphs depict a single, representative cohort.

2.5 Pacing

At the conclusion of the training period, 25-day old flies were removed from the study and subjected to electrical pacing as in Wessells et al (2004). Briefly, flies are placed between two electrodes touching conductive jelly spread over the electrodes and the heart is paced with a square wave stimulator at 40 V and 6 Hz for 30 s. The percentage of fly hearts that responded to pacing with either fibrillation or arrest was recorded as "% failure". Percent failure is a marker for stress sensitivity and characteristically declines with age (Piazza et al., 2009b; Wessells and Bodmer, 2004). Endurance exercise reduces cardiac failure rate across ages in trained male *Drosophila* (Piazza et al., 2009a; Sujkowski et al., 2015). Pacing experiments were performed in duplicate with $n \geq 68$ for all pacing experiments. Data were analyzed using chi-squared tests for probabilities with Yates' continuity correction. Tables and graphs depict a single, representative cohort.

2.6 Flight performance

Flight analysis was performed on day 25 after training was complete. Flight was analyzed as in Sujkowski et al. (2015). Triplicate cohorts of at least 71 flies were exercise trained in narrow vials housing groups of 20 age-matched siblings. Acrylic sheeting with paintable adhesive was placed in the flight tube, and fly cohorts were ejected into the apparatus to record flight performance and subsequent landing height after release. Fly cohorts were introduced to the flight tester one vial at a time using a gravity-dependent drop tube in order to reduce variability. After a full cohort of flies was captured on the adhesive, the sheeting was removed to a white surface in order to photograph landing height of each fly. Flies with

damaged wings were censored from final analysis to control for mechanical stress not related to training performance. Images were analyzed using ImageJ. Landing height graphs depict mean +/− SD with Tukey *post-hoc* test between all pairwise comparisons. Asterisks indicate significantly different groups. Tables represent 2-way ANOVA factoring nucleotype x training in all genotypes, and mitotype x nucleotype in trained and untrained groups separately. Tables and graphs depict a single, representative cohort.

2.7 Lysotracker

Similar to cardiac pacing and flight, Lysotracker staining of adult fat bodies was performed as in Sujkowski et al. on day 25 (2012a). Adult flies separated by treatment were dissected, ventral side up, in room temperature PBS. Partially dissected flies with their fat bodies exposed were rinsed 1X in fresh PBS. Lysotracker green (Molecular Probes, Eugene, OR) was diluted to 0.01μM in PBS and applied to dissected preps for 30 seconds. Samples were washed 3 times in fresh PBS. Stained fat bodies were subsequently removed and mounted in Vectashield reagent (Vector Laboratories, Burlingame, CA, USA). Confocal work was done at the Microscopy, Imaging and Cytometry Resources Core at Wayne State University, School of Medicine on a Zeiss Laser Scanning LSM 780 (Jena, Germany) using a 40X oil immersion objective. Images were analyzed using ImageJ (Bethesda, MD). 10 samples were analyzed for each sample and duplicate biological cohorts were assessed for each group. Lysotracker graphs depict mean +/− SEM with Tukey *post-hoc* test between all pairwise comparisons. Asterisks indicate significantly different groups. Tables represent 2-way ANOVA factoring nucleotype x training in all genotypes, and mitotype x nucleotype in trained and untrained groups separately. Tables and graphs depict a single, representative cohort.

2.8 Citrate Synthase Activity

Triplicate biological replicates of 8 age-matched adult male flies of each genotype were homogenized in 400 μL ice-cold Cellytic M buffer (Sigma Catalog Number C2978). Protein concentration of each sample was determined using BCA (Pierce BCA Protein Assay Kit (ThermoFisher cat. 23225) according to manufacturer's protocol with the following modification: The volume of homogenate pipetted from each biological replicate was reduced from 20 μL to 5 μL per well in order to stay within range of the standard curve. Sample volumes were adjusted so all had equal protein. Citrate Synthase activity was determined using the assay kit according to protocol (Sigma Catalog number CS0720). Briefly, an assay mix of 176 μL 1X assay buffer, 2 μL 10mM DNTB, 2 μL 30mM AcCoA and 10 μL sample was added per well and read on a kinetic program at 412 nm every 30 seconds for 4 minutes and 30 seconds to determine baseline. 10 μL 10 mM Oxaloacetate (made fresh in 1x assay buffer) was added to all wells, and the plate was read again as described above. Change in slope was calculated to determine activity/minute/mg of total protein.

2.9 Statistical analyses

The negative geotaxis (Climbing Speed) data were analyzed using mixed effect models in the R statistical package. The data reported in Table 1 were based on four replicate vials of 20 flies for each genotype. Each vial was quantified for climbing on successive days as

repeated measures. While individual flies were not quantified, the proportion of flies in each vial was quantified on successive days, so the vial is the unit of repeated measure. Because there were very few deaths in each vial, this is a more appropriate way to capture variation due to Age than to treat it as a survivorship analysis.

Statistical analyses followed two general three-way models:

Climbing Index ~ G + T + A + GxT + GxA + TxA + GxTxA + error, where G, T and A are the terms in the model for Genotype, Treatment (Exercised vs. Unexercised) and Age (different days as shown in Figure 1), respectively, plus all interaction terms.

We also separated the Nuclear and mtDNA components of Genotype in additional models that were run separately on the Exercised and Unexercised treatments:

Climbing Index ~ N + M + A + NxM + NxA + MxA + NxMxA + error, where N, M and A are the terms in the model for Nuclear genotype, mtDNA genotype and Age, respectively, plus all interaction terms.

To correct for the autocorrelation structure across the repeated measures of the Age effect in these three-way models, we used the R libraries car and nlme, and the gls and lme functions, with the autocorrelation correction as “correlation = corAR1(form = ~ Age |ReplicateVial)”. This treats the replicate vials as random effects with a lag time of 1, which captures the successive days of climbing analyses. A unique auto correlation value was estimated for each model and data set using the ACF function in R: ACF(model, form = ~1| ReplicateVial). The Results were summarized using the anova(model) and Anova(model) functions, which display F-values and Chi-Square tests, for analyses of variance, and deviance, respectively. The values reported for the analysis of deviance quantify the effects of comparing a fixed effect model to the model with the random effect of replicate vial corrected for autocorrelation. The R scripts describing these analyses are provided in the Supplemental material, and are modified from those reported by S. Mangiafico (http://rcompanion.org/handbook/I_09.html).

Statistical analyses for the data presented in Figures 3, 6 and 7 followed the same strategy with very similar models. The Ra / La lines are a matched set of genotypes where each mtDNA is represented on each Nuclear genome, so tests of Genotype can be partitioned orthogonally for tests of Nuclear x mtDNA interactions.

The mitoswitch lines include 10 genotypes, three of which are original isofemale lines (OreR, w^{1118} and Zim 53), and the w^{1118} mtDNA and the Zim53 nuclear genome are not paired with all other genotypes. Thus analyses were of two types: ANOVAs among the 10 Genotypes testing for interactions with Training effects, and ANOVAs for a subset of eight genotypes where four mtDNAs (*D. melanogaster* mtDNA *OreR* and *Zim53*, and *D. simulans* mtDNAs *sm21* and *siI*) are each paired with the two nuclear genomes (*D. melanogaster* *OreR* or w^{1118}). For these eight mitonuclear genotypes three-way ANOVAs were possible to test for Nuclear x mtDNA x Training interactions. Finally, within the eight orthogonal mitoswitch genotypes, two-way ANOVAs were performed separately for the Unexercised and Exercised samples, testing for Nuclear x mtDNA interactions.

Mitoswitch lines are analyzed twice in Table 2, once with only one repetition for each type, and another time with multiple repetitions of three groups pooled in the model. Thus, the degrees of freedom for each term does not change between the two analyses, but the total residual DF does. Both analyses gave qualitatively similar results.

For each phenotype, the following models were run in the R statistical package, using the aov and lm functions, and reporting results using the summary(model) and Anova(model) commands. Type II sum of squares were reported, but in most cases the data sets were balanced.

For the Ra/La and 10 mitoswitch lines, the following general 2-way model was tested Phenotype ~ G + T + GxT + error

For the orthogonal Ra/La and 8 mitoswitch lines, the following 3-way model was tested: Phenotype ~ N + M + T + NxM + NxT + MxT + NxMxT + error

And within either the Unexercised or Exercised samples of flies, the following 2-way model was tested: Phenotype ~ N + M + NxM + error.

In these models, G = the term for genotype (i.e., joint mito-nuclear genotype), T =the term for Training (Unexercised vs. Exercised) and M = the term for mtDNA (either Ra or La mtDNA), or one of the four mtDNAs from *D. melanogaster* or *D. simulans* stated above.

In each case, we test the hypothesis that Genotype, Training regimen, or Nuclear or mtDNA genotype explains significant levels of variation across treatments. Of additional interest is the strength of the interaction terms in these models, as they reflect the consistency, or context-dependence, of the main experimental variables we built in to this overall experiment. It should be noted that the data in Figure 3 appear as a time-course of survivorship format, but the data actually represent an attrition profile across 8 replicate vials, due to fatigue over time. As such, the vials were independent and were not treated as repeated measurements. Replicate sets of 8 vials of 20 flies for each genotype and Training treatment were subjected to climbing assays over the course of a given day. When fewer than 5 flies in a vial showed climbing activity, the time that vial was marked as ‘fatigued’ was taken as the response variable. Each ‘curve’ in Figure 3 has 8 points on it, representing the 8 initial vials and the time each one failed to climb. These time point data were normally distributed across the data set, and were treated as independent data observations in the ANOVAs described above.

ANOVAs are reported in Table 1 and 2, with the Ra/La lines and mitoswitch lines shown separately. Test results report Sum of Squares (Type II), F-value, and P-value, with the R-squared and associated degrees of freedom.

2.9 Data and Reagent Availability

All raw data and reagents will be made available to other researchers upon request.

3. Results

3.1 Longitudinal climbing performance

La flies are selectively bred for longevity from their parental *Ra* line (Arking, 2001; Arking et al., 2002; Arking et al., 1996). $RaLa_{(m)}$ and $LaRa_{(m)}$ flies are reciprocal isogenic lines containing heterologous mito-nuclear combinations, with the nucleotype indicated first followed immediately by mitotype, as indicated by the subscript (*m*) (Soh et al., 2007a).

La flies perform better than *Ra* flies in an acute test of climbing speed measured longitudinally across five weeks as reported previously (2-way ANOVA, genotype effect, $p<0.0001$) (Figure 1A) (Piazza et al., 2009a; Sujkowski et al., 2015). In all genotypes, climbing performance declines normally with age, but *Ra* flies respond to exercise with increased climbing speed relative to age-matched, unexercised siblings as previously observed (2-way ANOVA, exercise effect, $p\leq0.0273$). In contrast, age-matched *La* cohorts receive no further training benefit (Figure 1A), also observed previously (Sujkowski et al., 2015). $LaRa_{(m)}$ flies improve negative geotaxis speed in comparison to unexercised controls with exercise training (2-way ANOVA, exercise effect, $p<0.0001$) (Figure 1B). Similar to *La* flies, $RaLa_{(m)}$ lines show a reduced decline in negative geotaxis speed with age, resulting in enhanced climbing speed compared to *Ra* with or without training (2-way ANOVA, genotype effect, $p<0.0001$) (Figure 1B, compare 1A to 1B).

The next lines tested were three wild type strains of *D. melanogaster* with their own mtDNA (*Oregon R*, w^{1118} and *Zimbabwe 53*), as well as additional mito-switched lines with one of several types of mtDNA placed on to the *Oregon R* (*OreR*) or w^{1118} nuclear chromosomal backgrounds. Mitochondria were either from *D. simulans* (*siI*-from a Hawaii strain, or *sm21*-from strain C167.4 which is the *siI* haplotype) or from *D. melanogaster* (*OreR*, w^{1118}, or *Zimbabwe*, (*Zim53*). These lines are notated with the mitotype first, indicated by a subscript (*m*), followed by the nucleotype (Zhu et al., 2014). Both *OreR* and w^{1118} wild type flies improve negative geotaxis speed across ages with endurance exercise (2-way ANOVA, exercise effect, $p\leq0.0030$, $p\leq0.0280$, respectively), but *OreR* flies perform comparatively better and decline less rapidly with age than w^{1118}, independent of training status (2-way ANOVA, genotype effect, $p<0.0001$) (Figure 1C).

OreR flies with *sil* mitochondria (e.g., $siI_{(m)};OreR$) increase negative geotaxis speed across ages with endurance training (2-way ANOVA, exercise effect, $p\leq0.0081$), but do not reach improvement equivalent to *OreR* with matched mitochondria (2-way ANOVA, exercise effect, $p<0.0001$, genotype effect $p<0.0001$) (Figure 1D), *OreR* with *sm21* mitochondria, however, receive no benefit from exercise training, and even become a little slower (2-way ANOVA, exercise effect, $p\leq0.0276$) (Figure 1E). w^{1118} flies with *siI* mitochondria reduce speed with exercise but surpass performance of trained w^{1118} flies whether exercised or not after day 25 (2-way ANOVA, exercise effect, $p<0.0001$, genotype effect $p<0.0001$) (Figure 1F). w^{1118} flies with *sm21* mitochondria also fail to improve climbing speed with training but resemble untrained w^{1118} flies at young ages (2-way ANOVA, genotype effect, $p=0.2526$) and climb slightly better than wild-type untrained flies after day 20 (2-way ANOVA, genotype effect, $p\leq0.0471$) (Figure 1G). However, w^{1118} flies with *OreR* mitochondria respond to exercise with increased speed, similar to *OreR* (2-way ANOVA,

exercise effect, p≤0.0120) (Figure 1H), while w^{1118} flies with *Zim53* mitochondria had a modest response to exercise in week 1 only (2-way ANOVA, exercise effect, p<0.0001) (Figure 1H). Similarly, *Zim53 melanogaster* with their own mtDNA do not improve climbing speed with exercise, indeed performing worse at some individual time points (2-way ANOVA, exercise effect, p≤0.0478) and *OreR* flies with *Zim53* mitochondria also reduce climbing speed with training (2-way ANOVA, exercise effect, p<0.0001) (Figure 1I).

In order to better visualize the response to exercise, independent of differences in starting speed or changes with age, we graphed the difference between the speed of exercised and unexercised flies (from Figure 1) of the same subtype, at both the beginning and end of the exercise protocol. On day 5 of adulthood, *La* flies have higher negative geotaxis scores in an acute test of climbing speed than age-matched *Ra* flies (ANOVA with Tukey *post-hoc* test, p=0.0014) (Figure 2A), as previously reported (Piazza et al., 2009a; Sujkowski et al., 2015). Day-5 climbing speed of $RaLa_{(m)}$ flies is statistically indistinguishable from *La* cohorts of the same age, while $LaRa_{(m)}$ flies resemble *Ra* cohorts (Figure 2A). We next subjected *Ra, La,* $RaLa_{(m)}$ and $LaRa_{(m)}$ flies to our 3-week ramped endurance training protocol (Piazza et al., 2009a; Tinkerhess et al., 2012a). Exercised *Ra* flies increase climbing speed 12% relative to unexercised control *Ra* flies (Figure 2B). *La* flies, which have a much higher baseline speed, do not gain further additive benefit from training (Figure 2B). $LaRa_{(m)}$ flies show greater improvement in climbing speed than $RaLa_{(m)}$ cohorts after exercise (Figure 2B).

Thus, the unexercised speed of these lines is strongly predicted by their mitotype, while nucleotype also has a significant effect (Table 1). Because flies with the *Ra* mitotype increase their speed after training to match the flies with the high-speed *La* mitotype (Figure 1B, 2B), the effect of mitotype, and the nucleotype/mitotype interaction are only marginally significant after training (Table 1).

For the wild type and mitochondrial introgression strains, day 5 climbing speed was similar with the exception of *Zim53* wild type flies, which have increased negative geotaxis scores in comparison to all groups (ANOVA with Tukey *post-hoc* test, p≤0.0214) (Figure 2C). After 3 weeks of endurance exercise, both w^{1118} and *OreR* flies responded to training with increased climbing speed relative to unexercised control siblings (Figure 2D, E). In contrast, none of the mitochondrial introgressed lines increased post-training climbing speed in either the w^{1118} (Figure 2D) or *OreR* (Figure 2E) nuclear backgrounds. Thus, the results fall into two classes, with wild-type lines robustly responding to exercise with increased speed, and mitoswitched lines showing a blunted or absent response. This suggests that mito-nuclear compatibility plays an important role in modifying exercise-induced speed increases. Consistent with this observation, the nuclear x mito effect was significant in exercised and unexercised groups (Table 1).

3.2 Endurance

Endurance was measured using a fatigue tolerance assay in which flies are placed on the exercise machine in vials of 20 and allowed to run to exhaustion (Tinkerhess et al., 2012a). Vials are scored as fatigued when fewer than 5 flies remain running and data are analyzed similarly to a survival curve, referred to here as “runspan”. After 3 weeks of endurance exercise, trained *Ra* flies extended endurance in comparison to unexercised control *Ra*

siblings (log-rank, p<0.0001) (Figure 3A). *La* flies have enhanced post-training runspan whether exercised or not (log-rank, Ra UN vs La UN, p<0.0001, La UN vs La EX, p=0.6427) (Figure 3A), as previously observed (Sujkowski et al., 2015). $LaRa_{(m)}$ flies increase endurance after exercise training (log-rank, p<0.0001) (Figure 3B) while $RaLa_{(m)}$ flies have high endurance whether exercised or not (log-rank, $LaRa_{(m)}$ UN vs $RaLa_{(m)}$ UN, p<0.0001, $RaLa_{(m)}$ UN vs EX, p=0.6136) (Figure 3B). Thus, endurance either before or after exercise correlates strongly with the mitotype in these lines (Table 2), although the nucleotype also becomes significant in the exercised cohorts (Table 2).

OreR, w^{1118} and *Zim53* flies with matched nuclear and mitochondrial DNA increase endurance after exercise training relative to age-matched, unexercised siblings (Figure 3C, E, F, G, I). When *sm21 or sil* mitochondria are introduced into flies with *OreR* or w^{1118} nucleotype, the exercise response was severely blunted (Figure 3D–G). Flies with w^{1118} nucleotype and *OreR* mitotype had baseline endurance similar to *OreR*, but did not improve with exercise (Figure 3H). Flies with w^{1118} nucleotype and *Zim53* mitotype responded to exercise with improved endurance (log-rank, p=0.0094), but both pre- and post-exercise endurance were similar to w^{1118} and much lower than *Zim53* alone (compare Figure 3H to 3C and I). By contrast, flies with *OreR* mitotype and *Zim53* mitotype had high endurance, similar to the parental *Zim53*, but did not improve with exercise, even showing reduced endurance after training (log-rank, p=0.0090) (Figure 3I). The overall effect of nucleotype on endurance was much stronger than that of mitotype in the mitoswitch group (Table 2).

To better visualize exercise response independent of baseline endurance, we graphed the difference between maximum endurance of each line, before or after a three-week training program. The mitotype strongly predicted the endurance and the strength of the exercise effect in the closely related *Ra La* group (Figure 4A, B), but nucleotype was a better predictor of endurance in the more divergent mitoswitch group (Figure 4 C–E). This is likely due to the complex interaction between mitotype and exercise, where some mitotypes appear deleterious and others appear beneficial. Taken together, these results suggest that both mitotype and nucleotype play important roles in modifying endurance during training, and the relative strength of each role is context dependent.

3.3 Cardiac Performance

We have previously established that endurance exercise reduces cardiac failure in response to external electrical pacing (Piazza et al., 2009a; Sujkowski et al., 2015). External pacing is a cardiac stress assay that paces *Drosophila* hearts to twice their normal heart rate, then measures the percentage of flies that undergo arrest (Wessells and Bodmer, 2004), a phenotype that is highly age-dependent (Wessells et al., 2004) and acts as a marker for overall cardiac health. Failure rate in *OreR* and w^{1118} flies is normal at day 25 of adulthood. Endurance exercise exerts a protective effect on both lines, reducing the percentage of cardiac failure rate in response to external pacing (Figure 5A–D).

Flies with mitochondrial genotypes derived from *D. simulans* had varied responses to cardiac pacing following endurance exercise. $sil_{(m)}$;*OreR* flies did not improve cardiac performance with exercise training (Figure 5A, Chi-squared=0.1418, p=0.706479) Only $sm21_{(m)}$;*OreR* flies reduced pacing-induced cardiac failure after exercise training. (Figure

5B, Chi-squared=13.7221, p=0.0002). Cardiac failure in *siI*$_{(m)}$;*w*^{1118} flies was higher than average independent of training status (Figure 5C, Chi-squared=5.6004, p=0.0179). *sm21*$_{(m)}$;*w*^{1118} did not receive any cardiac benefit from endurance exercise, but had baseline cardiac performance that resembled trained *w*^{1118} control siblings whether exercised or not (Figure 5D, Chi-squared=0.2197, p=0.6329).

In contrast, flies with mitochondrial genotypes derived from *D. melanogaster* strains retained cardiac protection conferred by endurance training. Both *OreR*$_{(m)}$;*w*^{1118} and *Zim53*$_{(m)}$;*w*^{1118} lines reduced cardiac failure in response to pacing stress after exercise, (Chi-squared=20.1719, p<0.0001, Chi-squared=5.7736, p=0.0162, respectively). *Zim53* flies have a lower-than-average failure rate at day 25 of adulthood, and do not derive further benefit from exercise (Figure 5F, compare to *OreR, w*^{1118} EX) (*Zim53* UN vs *OreR* EX: Chi-squared=0.1479, p=0.7005, vs *w*^{1118} EX: Chi-squared=0.1841, p=0.6679) and *Zim53*$_{(m)}$;*OreR* have low cardiac failure rate whether exercised or not. (Figure 5F, compare to *OreR, w*^{1118} EX) (*Zim53*$_{(m)}$;*OreR* UN vs *OreR* EX: Chi-squared=0.4705, p=0.4927, vs *w*^{1118} EX: Chi-squared=3.5733, p=0.0587)

When considered across all lines tested in Figure 5, both nucleotype and mitotype had highly significant effects on cardiac pacing response (Table 3). The nucleotype had a stronger effect on post-training pacing response, although mitotype was also significant (Table 3). In general, these mitonuclear epistatic interactions are genotype-dependent. For example, the *sm21* mtDNA is responsive to training in the *OreR* nuclear background but not in the *w*^{1118} nuclear background (Figures 5B and D), but either of the *D. melanogaster* mtDNAs are responsive to training in the *w*^{1118} nuclear background (Figure 5E).

3.4 Flight performance

We have previously established that endurance training significantly improves flight index in wild-type *Drosophila* (Sujkowski et al., 2015). Both *OreR* and *w*^{1118} lines increase flight performance with exercise training (Figure 6A–D). Although *siI* mitotype flies in the *OreR* background do not improve landing height with exercise (Figure 6A), *siI* mitotype flies in the *w*^{1118} nuclear background have increased flight performace compared to the *w*^{1118} nucleotype in both unexercised (ANOVA with Tukey *post-hoc* test, p=0.0002) and exercised (ANOVA with Tukey *post-hoc* test, p<0.0001) groups (Figure 6C). Flies with the *sm21* mitotype fail to adapt to exercise training with increased landing height in either the *OreR* or *w*^{1118} nuclear background (Figure 6B, D). Neither *OreR*$_{(m)}$;*w*^{1118} nor *Zim53*$_{(m)}$;*w*^{1118} flies increase flight with exercise training (Figure 6E). *Zim53* lines have strong, exercise-independent flight performance, and *Zim53*$_{(m)}$;*OreR* flies improve flight performance with exercise (Figure 6F, ANOVA with Tukey *post-hoc* test, p<0.0001).

When mitoswitch groups were considered together, both nucleotype and mitotype were significant, although the interaction (nucleotype-by-mitotype) was highly significant only in post-training flight (Table 3).

3.5 Lysosomal activity

Exercise training increases Lysotracker staining in adipose tissue of wild-type male flies (Sujkowski et al., 2015; Sujkowski et al., 2012b). *siI*$_{(m)}$;*OreR, sm21*$_{(m)}$;*w*^{1118} and

siI$_{(m)}$;*w*^{1118} do not increase fat body lysosomal activity after exercise training as seen in exercised *OreR* and *w*^{1118} flies with matched nuclear and mtDNA (Figure 7A–C). Exercise-trained *sm21*$_{(m)}$;*w*^{1118} flies, however, upregulate fat body Lysotracker normally (Figure 7D, ANOVA with Tukey *post-hoc* test, p=0.0007). *OreR*$_{(m)}$;*w*^{1118} have low fat body Lysotracker staining, and *Zim53*$_{(m)}$;*w*^{1118} flies also show atypical Lysotracker staining (Figure 7G). *Zim53* and *Zim53*$_{(m)}$;*OreR* lines have low Lysotracker staining in adipose tissue whether or not they are exercise trained (Figure 7F). Both mitotype and nucelotype had significant effects on Lysotracker staining, although mitotype was much stronger (Table 3).

Mitochondrial Function—Because the effect of mitotype is strongest in the closely related *Ra* and *La* lines, we examined mitochondrial function in these lines before and after exercise. We were unable to detect significant differences that tracked with performance in pairwise comparisons for either ATP production, mtDNA/nuclear DNA ratio or in Complex II activity of isolated mitochondria (Supplemental Tables S1–S3). However, we found a strong difference in whole-fly citrate synthase activity between *Ra* and *La* mitotypes (Figure 8). This difference was independent of nucleotype and was affected by training only in the *Ra* mitotype (Figure 8), paralleling the climbing speed and endurance results (Figure 1,3).

Citrate synthase acitvity reflects increased TCA cycle activity and has been previously shown to increase in trained muscle of mammals (Ferreira et al., 2010; Li et al., 2011), without necessarily increasing mitochondrial number (Vigelso et al., 2014). Therefore, it seems plausible that increased citrate synthase activity in the *La* mitotype may have functional significance to the increased endurance of flies carrying this mitotype. Future work will be necessary to uncover the molecular mechanism by which the activity of this nuclear-encoded enzyme is modified by mitotype.

In summary, mitotype played its strongest role in the closely related *Ra* and *La* lines, but was also significant in the more divergent mitoswitch lines. Among the more divergent lines, the *D. melanogaster Zim53* and *D. simulans sil* mitotypes demonstrate a clear influence on performance that the *D. simulans sm21* mitotype does not, despite the fact that *Zim53* and *siI* come from different species and *sm21* and *siI* are more similar in mtDNA sequence. This suggests that specific sequences within the mtDNA are likely to be important, as degree of divergence by itself does not fully explain the observed mitonuclear interactions. The upregulation of citrate synthase activity in a high-performance mitotype suggests that upregulation of TCA cycle activity is likely to be a key downstream effect of the relevant mtDNA sequences.

4. Discussion

4.1 Mitochondria and exercise training

Recent findings in several organisms, including humans (Irrcher et al., 2003; Powers et al., 2014; Yan et al., 2012), mice (Lantier et al., 2014; Lira et al., 2013; Matsakas et al., 2010), *Drosophila* (Laker et al., 2014b; Piazza et al., 2009a) and *C. elegans* (Laranjeiro et al., 2017; Restif et al., 2014) have supported the idea that chronic endurance exercise increases mitochondrial health. It has been previously observed that strains with nucleotype and mitotype derived from different progenitor strains have profound alterations in the dietary

effects on longevity and development time in *Drosophila* (Mossman et al., 2016; Zhu et al., 2014) and on metabolism and aging in mice (Latorre-Pellicer et al., 2016). Here, we examine the idea that replacement of mitochondria with exogenous mitochondria derived from other strains or other species would confer changes in exercise capacity.

The strains used have widely divergent baseline exercise capacities, with *La* having the highest and w^{1118} the lowest among them. *Zim53* has an unusual profile, with a baseline capacity similar to *OreR*, but with a slower age-related decline that is not responsive to exercise training. Indeed, the *Zim53* group behaved as an outlier in almost every assay.

We find that exogenous mitochondria can, in fact, change the baseline capacity of a given strain (*e.g.* $RaLa_{(m)}$). However, in other cases, baseline capacity is unaltered by introduction of exogenous mitochondria (*e.g.* $OreR_{(m)};w^{1118}$). Despite their divergent baseline capacities, all the wild-type strains carrying their own mitochondria responded to exercise training with the characteristic changes to speed, endurance, etc.

We find a general trend that strains with exogenous mitotypes have a reduced quantitative response to exercise training in several assays, including speed, endurance, cardiac stress resistance, and adipose Lysotracker staining. The fact that exercise response is more negatively affected by exogenous mitochondria than baseline capacity suggests that mito-nuclear compatibility is an important factor during exercise adaptations. This seems to be the case even when baseline capacity is not altered. Because chronic exercise induces mitochondrial biogenesis and mitophagy, it is likely that incompatibilities that may be innocuous in sedentary animals are highlighted under conditions where mitochondria are undergoing active replication and fission. Consistent with this idea, variation in mitochondrial *tRNA* sequence has been identified as a molecular mechanism for mito-nuclear incompatibility in temperature adaptation (Hoekstra et al., 2013; Zhang et al., 2017).

Despite the evident importance of mitotype as a determinant of exercise capacity and response to exercise training, it is clear that the nuclear genome also has an important role to play. Microarrays have identified conserved pathways that are altered transcriptionally by exercise in mice (Ort et al., 2007; Teran-Garcia et al., 2005) and *Drosophila* (Sujkowski et al., 2015). Furthermore, conserved single-gene candidates have been identified that are capable of conferring benefits of exercise, including *PGC1-α* (Diop et al., 2015; Tinkerhess et al., 2012b; Xiong et al., 2015), as well as invertebrate-specific factors, such as *Mthl-3* in *Drosophila* (Sujkowski et al., 2015). Epigenetic markers have also been linked to exercise, including markers that can be passed by maternal heredity in mice (Laker et al., 2014a).

Different assays clearly showed different sensitivity to mitotype and nucleotype across cohorts. For example, lysosomal activity was more sensitive to mitotype, whereas endurance in mitoswitch groups was more sensitive to nucleotype. The variety of effects clearly suggest that systemic adaptation to increased daily exercise involves multiple interactions between the mitochondrial and nuclear genomes in various tissues. Thus, it seems clear that nuclear factors, mitochondrial factors, and the interactions between them are all of importance in driving the exercise response.

4.2 Mitochondria from selected lines, wild strains and divergent species

The clearest effect of mitotype on exercise capacity and exercise response was seen in the lines derived from *Ra* and *La*. One potentially important difference between these lines and the others used in the study is that these lines share a common progenitor, as *La* was created by selection for longevity from the original *Ra* (Arking, 1987). Thus, they are more closely related than any other combination used here. Previous work has demonstrated that mitochondria from *La* flies predict the longevity of the line under dietary restriction (Soh et al., 2007b), suggesting that mitochondrial genes, whether nuclear or mitochondrially encoded, are part of the selection effect. It is further notable that the introgression of a mtDNA from a different species (*D. simulans* mtDNA in *D. melanogaster* chromosomes) does not produce a consistently more dysfunctional fly genotype, as might be expected from the breakdown of a co-adapted mitonuclear genetic interaction. Most of the variation across mtDNAs in baseline performance or response to exercise was attributable to variation between mtDNA within a species (*Zim53* vs. *OreR*, or *siI* vs. *sm21*). These findings are consistent with fitness assays using many of these same mitonuclear genotypes (Montooth et al., 2010), and a larger set of independent mtDNA introgression strains (Mossman et al. 2016). The *siI* and *Zim53* mtDNAs were more likely to contribute beneficial effects, and there was some indication that this was more pronounced in the w^{1118} nuclear background, than in the *OreR* nuclear background (see Figures 3, 4 and 6). This is consistent with other studies of mitonuclear epistatic interactions, where beneficial and deleterious combinations are common, but not predictable by the main effects of either nuclear or mitochondrial genome.

Microarray experiments demonstrated that 65% of the transcriptional changes between *Ra* and *La* are identical to the changes between *Ra* and exercised *Ra* (Sujkowski et al., 2015). Thus, the selection process that created the *La* line was inadvertently similar to the process of exercise-training. This raises the fascinating idea that introgressed mitochondria from the *La* line may be functionally equivalent to introducing mitochondria from an exercise-trained *Ra* back into a sedentary *Ra* fly. The common origin between the mitotype and nucleotype of the $RaLa_{(m)}$ and $LaRa_{(m)}$ may be reflected in better mito-nuclear compatibility, allowing the effect of the mitochondria to be more clearly demonstrated.

4.3 Gene x gene x environment interactions

Interactions between mitochondrial and nuclear genome have been demonstrated to play an important role in the response to dietary restriction (Mossman et al., 2016; Zhu et al., 2014) and hypoxia (Mossman et al., 2017) in *Drosophila*. Here, we find further evidence that major environmental changes, such as chronic endurance exercise, are dependent on interactions between the mitochondrial and nuclear genomes.

A growing body of literature has focused on individual differences in exercise response, both in model organisms (Britton and Koch, 2001; Koch et al., 2012; Mendez et al., 2016) and in humans (Bouchard et al., 2012; Puthucheary et al., 2011). While these differences are presumed to derive from genetics, relatively few conserved single genes have been identified that promote efficient exercise adaptations (Bostrom et al., 2013). Of those that have been identified, such as *PGC1-α* (Laker et al., 2014a; Tinkerhess et al., 2012b), a common thread

is regulation of either mitochondrial biogenesis or autophagy/mitophagy. As these processes require cooperation between mitochondrial and nuclear genes, it seems likely that mito-nuclear interactions are an important factor in the efficiency of individual exercise adaptation across the animal kingdom. Given the increased interest in mitochondrial replacement therapy for the treatment of mtDNA-encoded disease, the unpredictable nature of the outcomes in these experiments indicates that further study is needed to identify the mechanisms underlying high-fitness mitonuclear interactions.

While great progress has been made using model organisms to better understand responses to diet (Mossman et al., 2016; Ort et al., 2007; Rera et al., 2011), the response to chronic activity is much less well understood. Like diet, exercise is an important environmental variable with broad effects on metabolism and physiology. Now that multiple exercise models have been developed in *Drosophila* (Mendez et al., 2016; Piazza et al., 2009a; Tinkerhess et al., 2012a) and *C. elegans* (Laranjeiro et al., 2017), the stage is set to better explore these interactions using the strengths of intertebrate genetics.

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Highlights

- Introgression of mitochondria from different backgrounds or species allows separation of phenotypic contributions of mitotype and nucleotype
- Mitotype and nucleotype play significant roles in exercise capacity and adaptation to chronic exercise
- Mitotype from closely related species drives exercise capacity and ability to adapt to endurance training in Drosophila
- Mitotypes that favor increased exercise performance promote increased citrate synthase activity

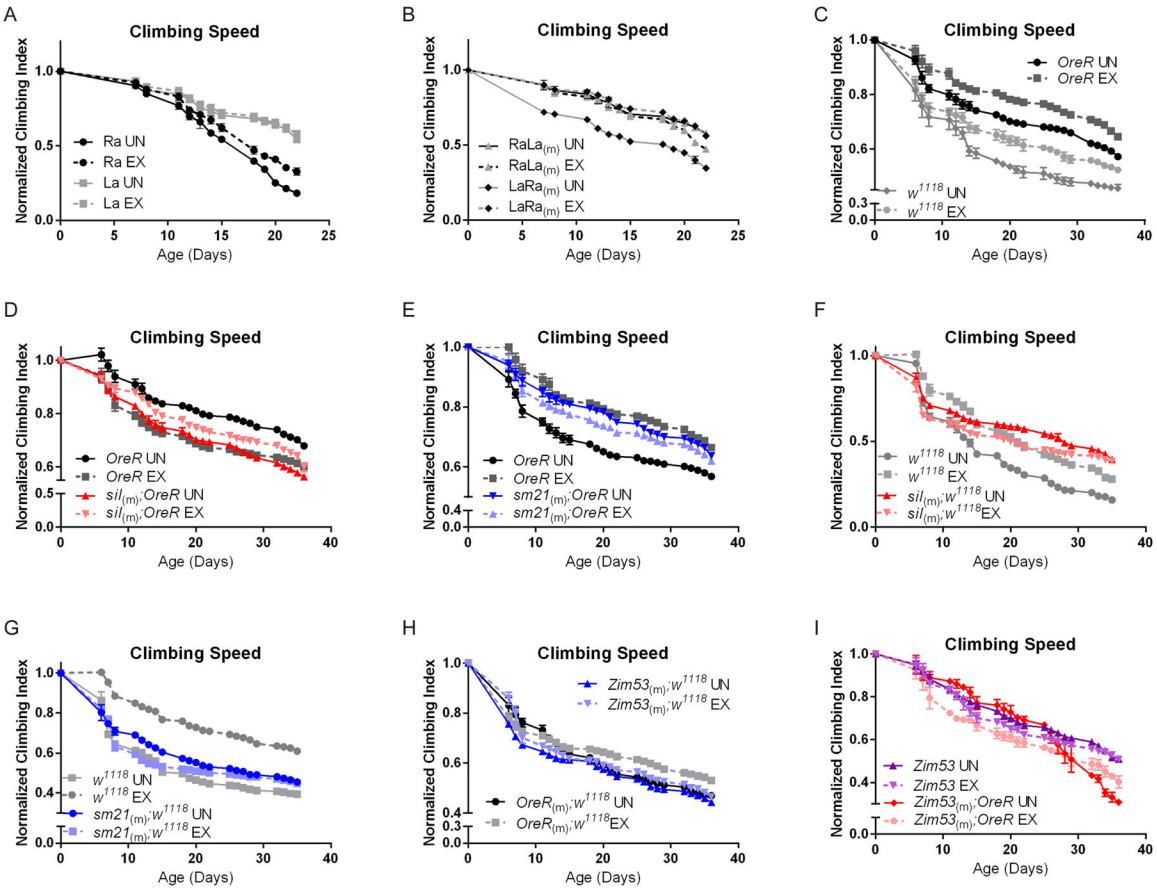

Figure 1: Mito-nuclear interactions differentially modulate climbing speed during endurance exercise

(A) Exercised (EX) *Ra* flies are protected against declining negative geotaxis speed with age compared to unexercised (UN) siblings. *La* flies have higher negative geotaxis speed than *Ra*. (B) Exercised $LaRa_{(m)}$ flies are protected against declining negative geotaxis speed with age compared to unexercised siblings. $RaLa_{(m)}$ flies have higher negative geotaxis speed than $LaRa_{(m)}$. (C) Both exercised *OreR* and w^{1118} flies are protected against declining negative geotaxis speed and *OreR* flies perform better than w^{1118} flies whether exercised or not. (D) $sil_{(m)}$;*OreR* flies are protected against declining negative geotaxis speed compared to unexercised siblings, but do not reach performance equal to exercised *OreR* flies. (E) $sm21_{(m)}$;*OreR* flies do not enhance negative geotaxis speed with training, and resemble trained *OreR* lines. (F) $sil_{(m)};w^{1118}$ lines reduce climbing speed with exercise in weeks 2–4 relative to unexercised siblings, but climb faster than trained w^{1118} flies after day 25.(G) Exercised $sm21_{(m)};w^{1118}$ flies reduce climbing speed in weeks 2 and 3 compared to untrained siblings, and perform better than untrained w^{1118} flies in weeks 3 and 4. Climbing speed does not reach performance of trained w^{1118} flies with matched nuclear and mtDNA. (H) $OreR_{(m)};w^{1118}$ flies are protected against declining negative geotaxis speed with age compared to unexercised siblings, but $Zim53_{(m)};w^{1118}$ lines are not. (I) Unexercised $Zim53_{(m)}$;*OreR* flies have increased climbing speed in the first 3 weeks of adulthood before a rapid decline in performance. Exercised $Zim53_{(m)}$;*OreR* flies climb significantly more slowly than unexercised siblings until week 5. *Zim53* flies do not improve climbing speed

with exercise training. n≥100 for all climbing experiments. Graphs are representative of a single repetition of at least duplicate cohorts. Error bars indicate SEM. ANOVAs reporting main and interaction effects are presented in Table 1

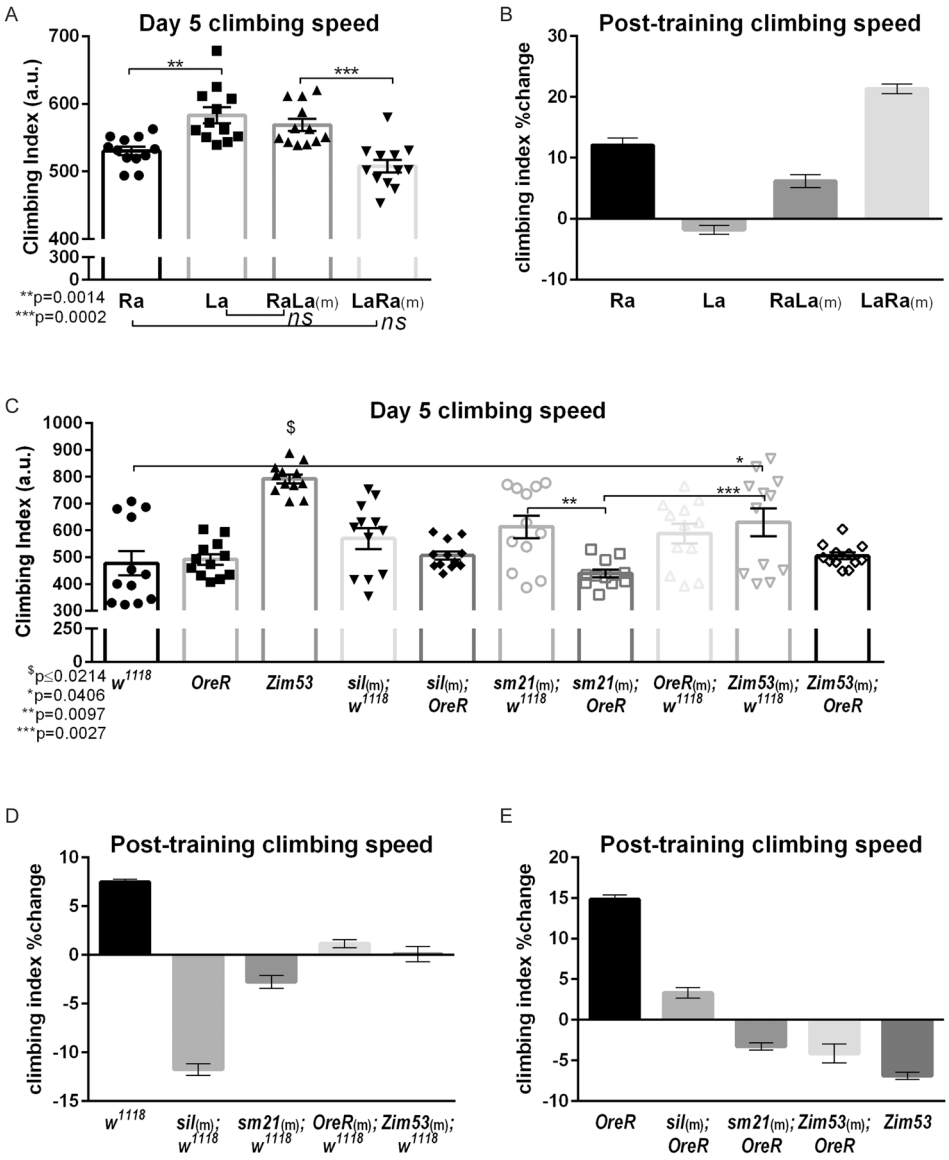

Figure 2: Acute climbing speed is affected by both nuclear and mitochondrial genotype in *Drosophila*

(A) *La* flies have higher climbing index in comparison to the parental *Ra* line at day 5 of adulthood. *RaLa*$_{(m)}$ flies have higher climbing index relative to *LaRa*$_{(m)}$ flies and perform similarly to *La* flies. *LaRa*$_{(m)}$ and *Ra* flies climb with similar speed (p=0.3361). (B) After exercise training, *Ra, RaLa*$_{(m)}$ *and LaRa*$_{(m)}$ flies all improve climbing speed in comparison to untrained control siblings. (C) At day 5 of adulthood, w^{1118}, *OreR, sil*$_{(m)}$;w^{1118}, *sil*$_{(m)}$;*OreR, sm21*$_{(m)}$;*OreR, OreR*$_{(m)}$;w^{1118}, *and Zim53*$_{(m)}$;*OreR* lines climb with similar performance (p=0.2320), but *Zim53* climbing index is enhanced in comparison to each group. Additional statistically significant pairwise comparisons are indicated with brackets. Following endurance exercise, only w^{1118} (D) and *OreR* (E) increase climbing speed across ages relative to untrained siblings. n≥100 for all negative geotaxis experiments. Graphs are representative of a single repetition of at least duplicate cohorts for all experiments presented

in the manuscript. Error bars indicate SEM. . ANOVAs reporting main and interaction effects are presented in Table 1

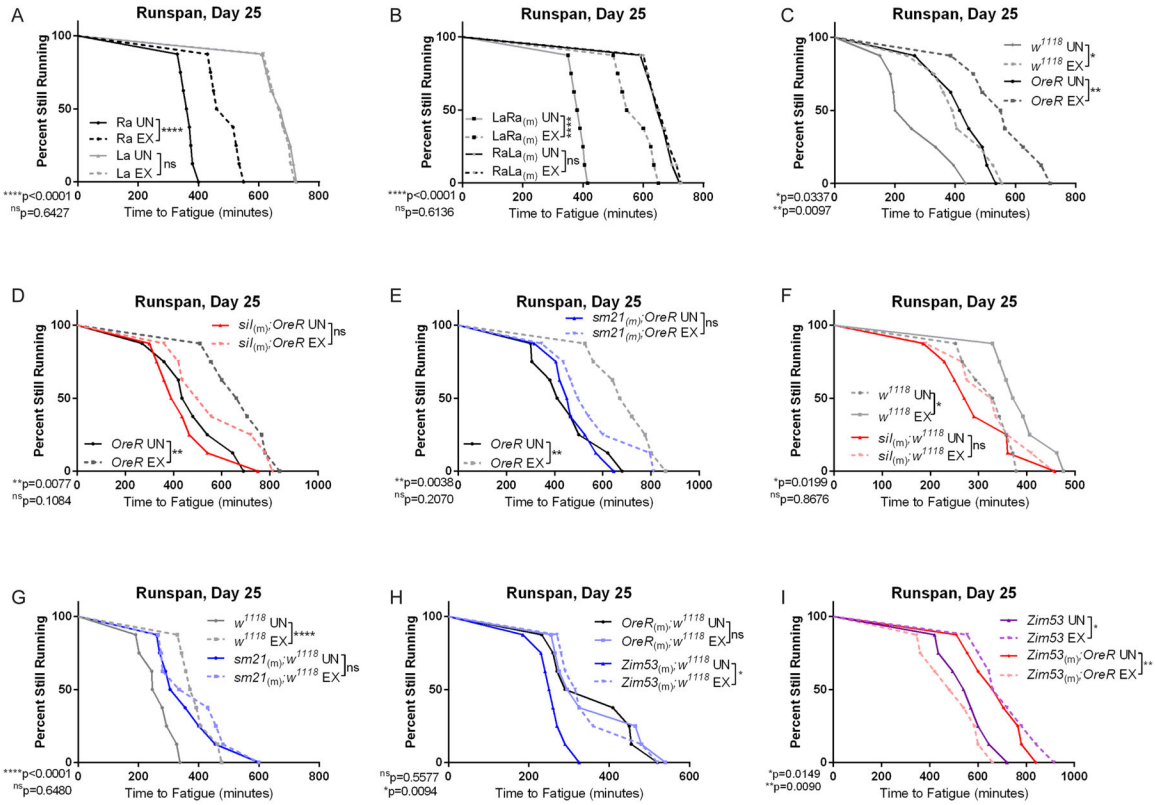

Figure 3: Exercise training increases endurance in *Drosophila* with matched nuclear and mitochondrial genotypes

(A) *Ra* flies increase endurance after exercise (EX) training. *La* flies have increased endurance in comparison to trained and untrained (UN) parental *Ra* flies, but do not receive further benefit from exercise. (B) $LaRa_{(m)}$ flies increase endurance after exercise training, while $RaLa_{(m)}$ flies have enhanced endurance independent of training status. (C) w^{1118} and *OreR* flies have better endurance after exercise training, and untrained *OreR* flies have higher runspan than untrained w^{1118} flies (p=0.0103), and trained *OreR* flies outperform trained w^{1118} cohorts (p=0.0150). Neither $siI_{(m)}$;*OreR* (D) nor $sm21_{(m)}$;*OreR* (E) increase endurance with exercise training like *OreR* flies with matched nuclear and mtDNA. Similarly, $siI_{(m)};w^{1118}$ (F) and $sm21_{(m)};w^{1118}$ flies (G) do not improve endurance with exercise training like w^{1118} flies (F,G). (H) $Zim53_{(m)};w^{1118}$ lines improve endurance with exercise training, but $OreR_{(m)};w^{1118}$ flies do not. (I) Untrained $Zim53_{(m)}$;*OreR* flies have greater endurance than trained siblings, but exercised *Zim53* flies improve endurance after exercise. n=8 vials of 20 flies for all endurance experiments. Graphs are representative of a single repetition of at least duplicate cohorts. p-values are determined by log-rank. ANOVAs reporting main and interaction effects are presented in Table 2

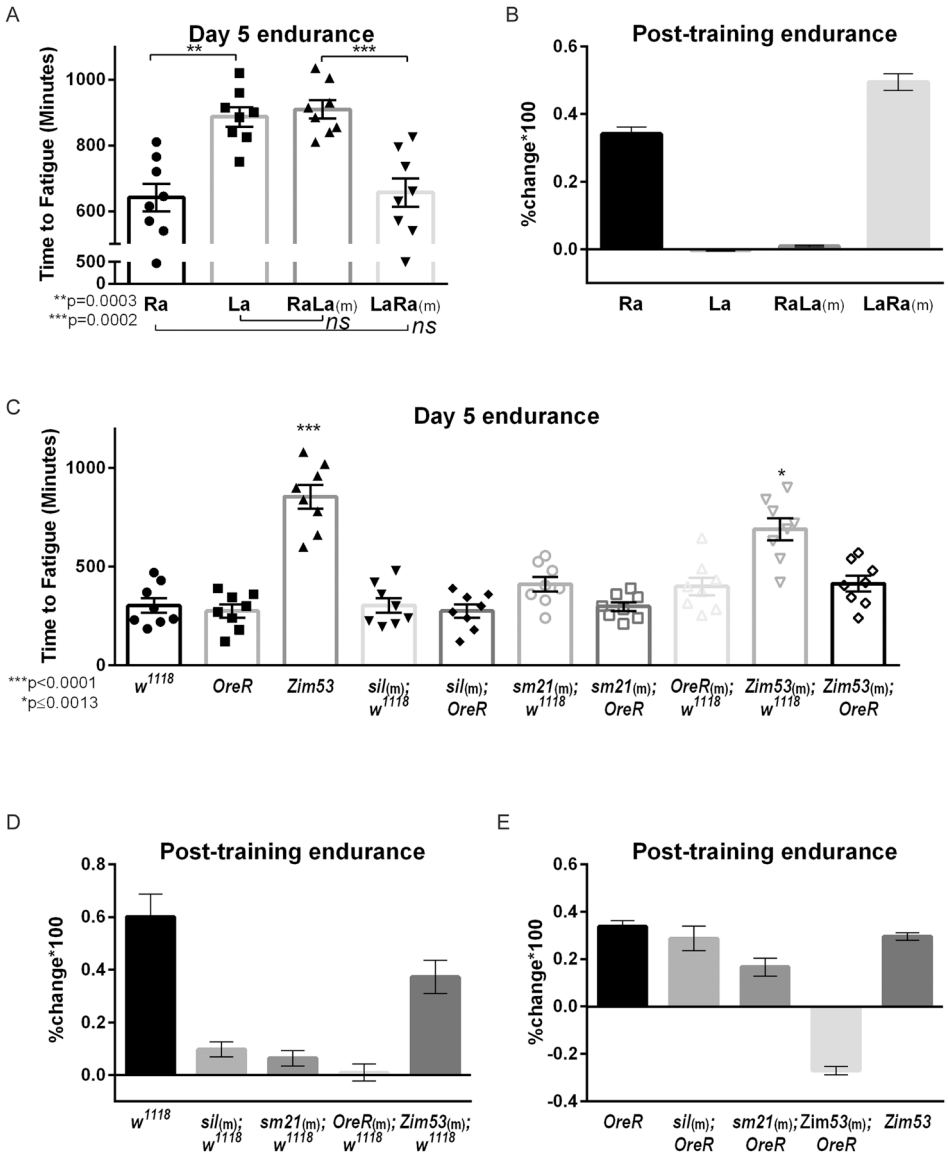

Figure 4: Exercise increases endurance independent of mito-nuclear mismatch

(A) *La* flies have increased endurance in comparison to the parental *Ra* line at day 5 of adulthood. $RaLa_{(m)}$ flies have better endurance than $LaRa_{(m)}$ flies and perform similarly to *La* flies (p=0.9707). $LaRa_{(m)}$ and *Ra* flies have equivalent endurance (p=0.9909). (B) After exercise training, *Ra* and $LaRa_{(m)}$ flies improve endurance compared to untrained cohorts. (C) At day 5 of adulthood, w^{1118}, *OreR*, $siI_{(m)};w^{1118}$, $siI_{(m)}$;*OreR*, $sm21_{(m)};w^{1118}$, $sm21_{(m)}$;*OreR*, $OreR_{(m)};w^{1118}$, and $Zim53_{(m)}$;*OreR* lines have similar endurance (p=0.1140), but $Zim53_{(m)};w^{1118}$ and *Zim53* have comparatively better endurance. Following endurance exercise, the majority of w^{1118} (D) and *OreR* mitotypes (E) increase endurance in comparison to unexercised siblings, with the exception of $OreR_{(m)};w^{1118}$ (D) and $Zim53_{(m)}$;*OreR* (E). n=8 vials of 20 for all endurance experiments. Graphs are representative of a single repetition of at least duplicate cohorts for all experiments presented in the manuscript. Error bars indicate SEM.

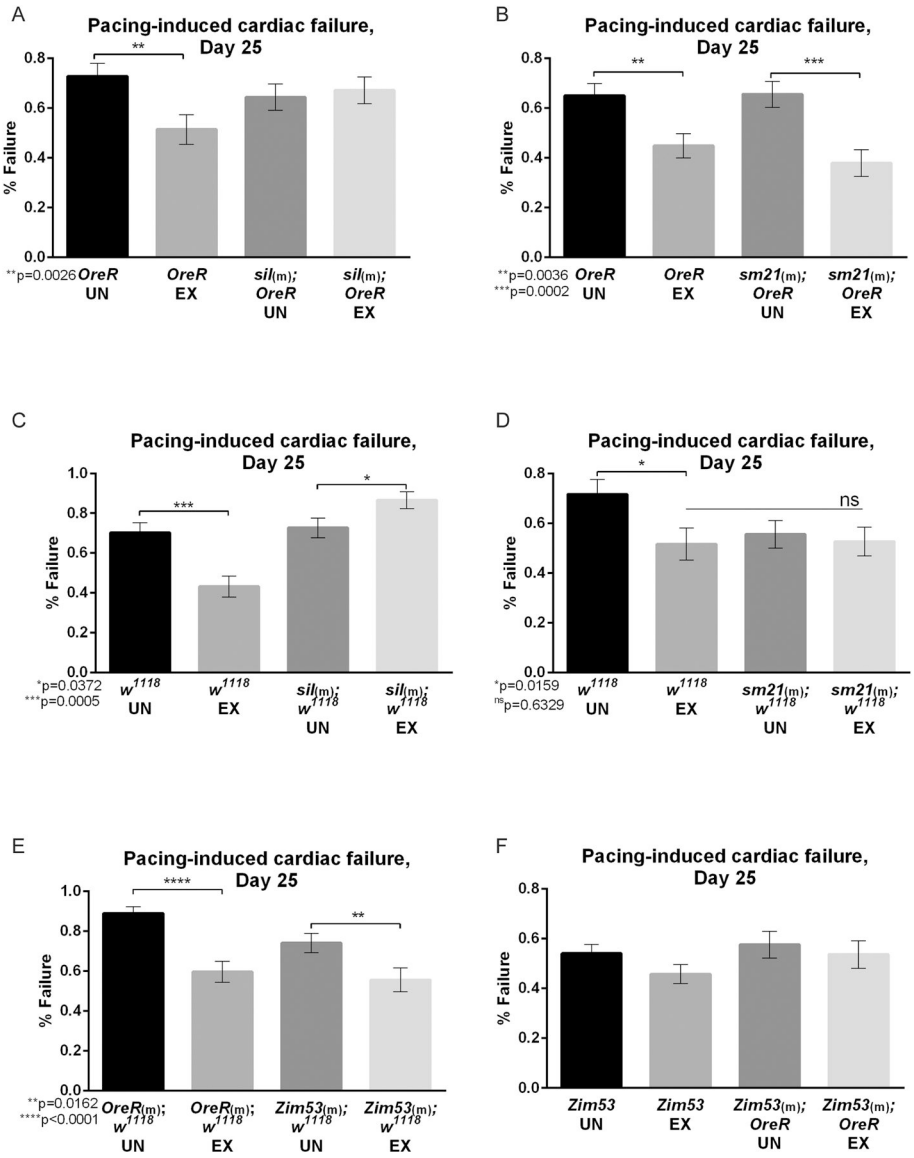

Figure 5: Mito-nuclear incompatibility negatively impacts cardiac health

(A) Exercise (EX) significantly reduces pacing-induced cardiac failure in *OreR* and *sm21*$_{(m)}$;*OreR* flies (B) in comparison to age-matched unexercised (UN) siblings, but *siI*$_{(m)}$;*OreR* flies (A) do not receive cardiac benefits from endurance exercise. (C) *siI*$_{(m)}$;w^{1118} flies do not receive cardiac protection from endurance training and have unusually high pacing-induced cardiac failure rate. (D) *sm21*$_{(m)}$;w^{1118} flies had significantly less cardiac failure than age-matched unexercised w^{1118} flies whether exercised or not. Exercised w^{1118} flies received cardiac protection from pacing-stress post-training (C, D). (E) Both *OreR*$_{(m)}$;w^{1118} and *Zim53*$_{(m)}$;w^{1118} flies had reduced cardiac failure compared to age-matched unexercised siblings and (F) *Zim53* and *Zim53*$_{(m)}$;*OreR* flies had low cardiac failure in response to pacing whether exercised or not. n≥67 for all pacing experiments. Graphs are representative of a single repetition of at least duplicate cohorts. p-values

generated by Chi-squared analysis, error bars indicate SEM. ANOVAs reporting main and interaction effects are presented in Table 3.

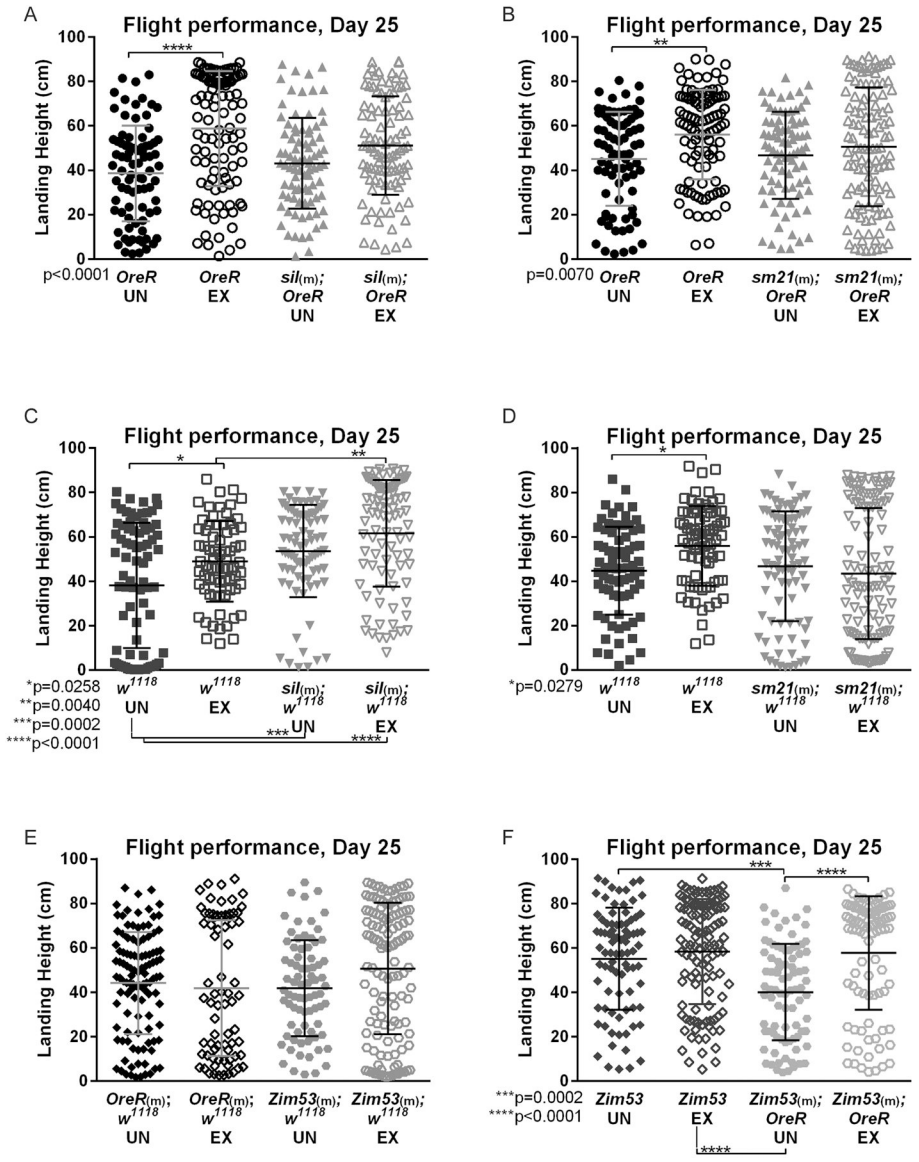

Figure 6: Enhancements in flight performance are weakly affected by mito-nuclear interactions

(A-D) Both *OreR* and w^{1118} flies improve landing height after endurance exercise (EX). (A) $sil_{(m)}$;*OreR*, (B) $sm21_{(m)}$;*OreR*, and (D) $sm21_{(m)}$;w^{1118} and fail to improve flight performance after endurance exercise. (C) $sil_{(m)}$;w^{1118} flies have enhanced flight compared to trained and untrained (UN) w^{1118} lines whether exercised or not. (E) Neither $OreR_{(m)}$;w^{1118} nor $Zim53_{(m)}$;w^{1118} flies increase flight performance after exercise training. *Zim53* flies have training-independent enhanced flight performance, and exercised $Zim53_{(m)}$;*OreR* flies increased landing height compared to unexercised control siblings. n≥71 for all cohorts. p-values generated by ANOVA with Tukey *post-hoc* comparison, error bars indicate SD. Graphs are representative of a single repetition of at least duplicate cohorts. ANOVAs reporting main and interaction effects are presented in Table 3.

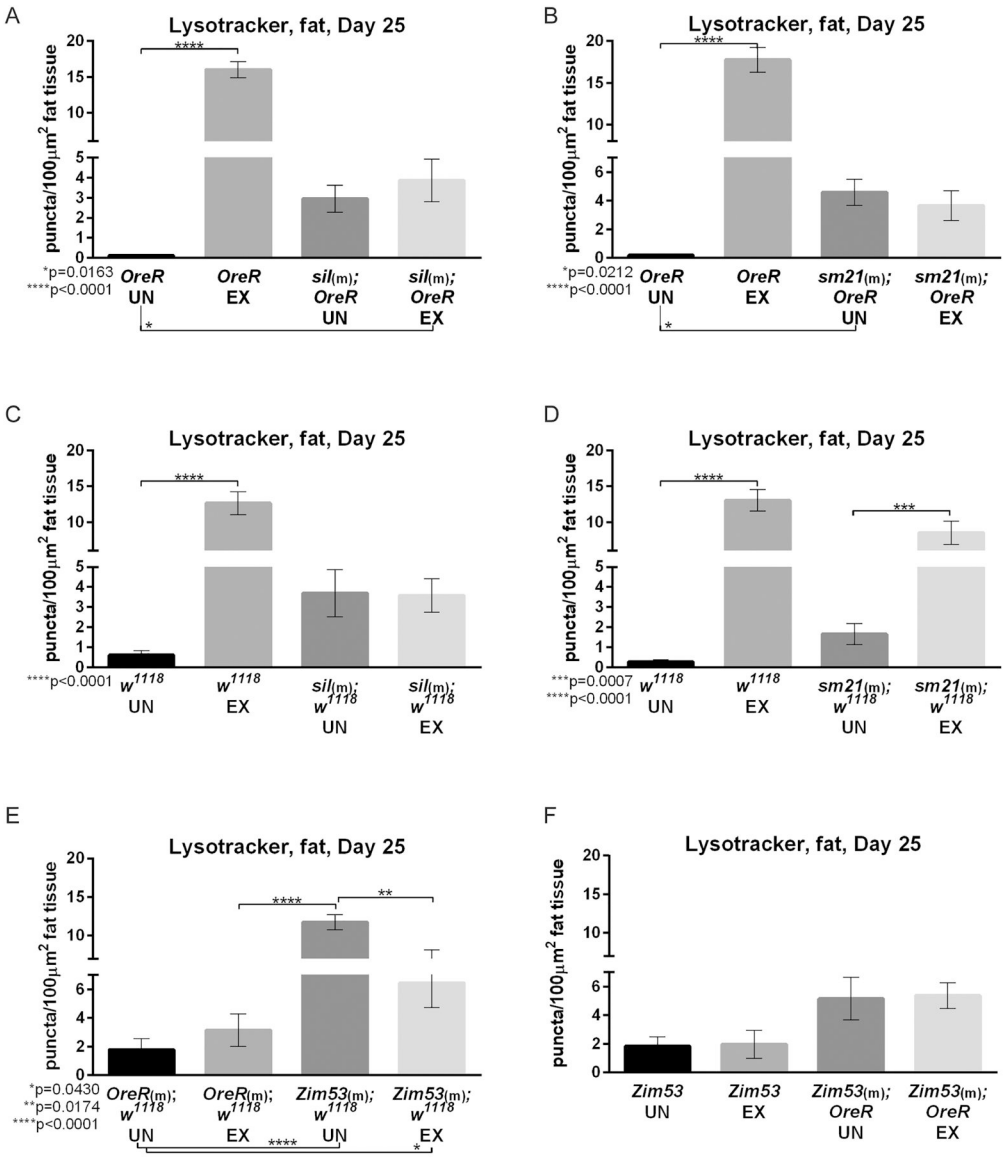

Figure 7: Mito-nuclear interactions strongly affect lysosomal activity in *Drosophila* fat body after endurance exercise

(A) *OreR* and w^{1118} flies upregulate fat body lysosomal activity after endurance training (EX), but in $sil_{(m)}$;*OreR* (A), $sm21_{(m)}$;*OreR* (B), and $sil_{(m)}$;w^{1118} flies (C) lysosomal activity in the fat body remains low. (D) Only $sm21_{(m)}$;w^{1118} flies have increased fat body Lysotracker staining after endurance exercise. (E) Similarly, $OreR_{(m)}$;w^{1118} and $Zim53_{(m)}$;w^{1118} do not upregulate fat body lysosomal activity after exercise. In fact, Lysotracker is higher in unexercised (UN) $Zim53_{(m)}$;w^{1118} flies than in exercised siblings. (F) *Zim53* and $Zim53_{(m)}$;*OreR*, flies have low Lysotracker staining in the fat body whether exercised or not. n=10 for all cohorts. p-values generated by ANOVA with Tukey *post-hoc* comparison, error bars indicate SEM. Graphs are representative of a single repetition of at least duplicate cohorts. ANOVAs reporting main and interaction effects are presented in Table 3.

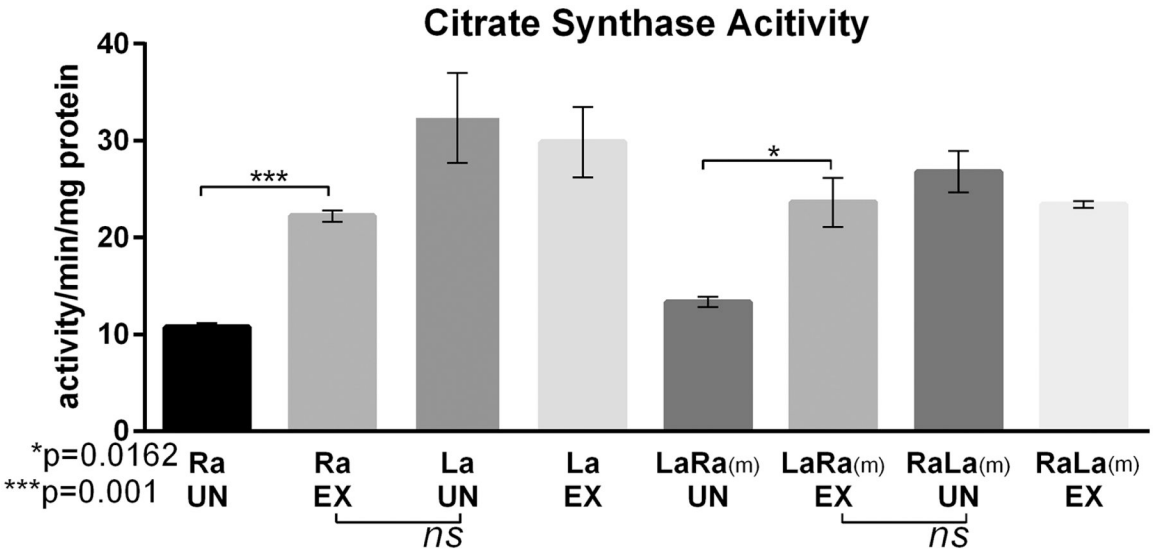

Figure 8: Citrate Synthase Specific Activity parallels climbing speed and endurance in closely-related *Ra* and *La* lines.

Trained *Ra* flies have higher citrate synthase activity than age-matched, untrained siblings. Untrained *La* flies have high citrate synthase activity that does not increase with training. $LaRa_{(m)}$ flies have increased citrate synthase activity after endurance training, similar to *Ra* flies, and $RaLa_{(m)}$ flies have citrate synthase activity similar to *La* lines independent of training status. n=8 flies for each cohort. p-values are generated by ANOVA with Bonferroni *post-hoc* comparison, error bars indicate SEM. Graph is representative of triplicate repetitions of triplicate biological cohorts.

Table 1

Repeated measures analysis of climbing speed by genotype, training and age

Ra/La			**Analysis of variance**		**Analysis of Deviance**	
Combined	Term in models	DF	F-value	p-value	Chisq	Pr(>Chisq)
	(Intercept)	1	21709.05	<.0001		
	Genotype	3	30.75	<.0001	92.24	<2.2E-16
	Training	1	23.60	<.0001	23.60	1.184E-06
	Age	1	1842.70	<.0001	1842.70	<2.2E-16
	Genotype:Training	3	14.99	<.0001	44.98	9.344E-10
	Genotype:Age	3	34.56	<.0001	103.67	<2.2E-16
	Training:Age	1	8.35	0.0041	8.35	0.0038662
	Genotype:Training:Age	3	6.46	0.0003	19.37	0.0002298
	Residuals	400				
Unexercised		DF	F-value	p-value	Chisq	Pr(>Chisq)
	(Intercept)	1	9584.20	<.0001		
	Nuclear	1	0.01	0.9237	0.01	9.236E-01
	mtDNA	1	99.86	<.0001	99.85	<2.2E-16
	Age	1	1009.07	<.0001	1009.07	<2.2E-16
	Nuclear:mtDNA	1	0.09	0.763	0.09	7.627E-01
	Nuclear:Age	1	5.54	0.0195	5.54	1.855E-02
	mtDNA:Age	1	64.36	<.0001	64.36	1.038E-15
	Nuclear:mtDNA:Age	1	10.89	0.0011	10.89	0.0009668
	Residuals	200				
Exercised		DF	F-value	p-value	Chisq	Pr(>Chisq)
	(Intercept)	1	12346.02	<.0001		
	Nuclear	1	23.20	<.0001	23.20	1.464E-06
	mtDNA	1	5.15	0.0244	5.14	0.023317
	Age	1	837.15	<.0001	837.15	<2.2E-16
	Nuclear:mtDNA	1	4.69	0.0316	4.69	0.030367
	Nuclear:Age	1	26.95	<.0001	26.95	2.09E-07
	mtDNA:Age	1	7.24	0.0077	7.24	7.115E-03
	Nucleanmt:DNAAge	1	6.59	0.011	6.59	1.023E-02
	Residuals	200				
Percent Change (Figure 2A)		DF	F-value	Sum Sq	Pr(>F)	
	Nuclear	1	0.44	8.1	0.51	
	mtDNA	1	228.22	4225.8	<2.2E-16	
	Nuclear:mtDNA	1	80.22	1485.3	1.62E-13	
	Residuals	76		1407.20		

Residual standard error: 4.303 on 76 degrees of freedom
Multiple R-squared: 0.8025,
Adjusted R-squared: 0.7947
F-statistic: 103 on 3 and 76 DF, p-value: < 2.2e-16

Mitoswitch			**Analysis of variance**		**Analysis of Deviance**	
10 Genotypes	Term in models	DF	F-value	p-value	Chisq	Pr(>Chisq)
	(Intercept)	1	25807.94	<.0001		
	Genotype	9	16.45	<.0001	157.34	<2.2E-16
	Training	1	0.88	0.3485	0.88	3.483E-01
	Age	1	2900.16	<.0001	2900.16	<2.2E-16
	Genotype:Training	9	1.31	0.228	11.68	2.322E-01
	Genotype:Age	9	9.70	<.0001	87.27	5.725E-15
	Training:Age	1	3.20	0.074	3.19	7.388E-02
	Genotype:Training:Age	9	0.55	0.8362	4.98	8.365E-01
	Residuals	1864				
8 Genotypes		DF	F-value	p-value	Chisq	Pr(>Chisq)
	(Intercept)	1	20061.80	<.0001		
	Genotype	7	19.73	<.0001	147.09	< 2.2E-16
	Training	1	0.32	0.5708	0.32	5.707E-01
	Age	1	2224.39	<.0001	2224.39	< 2.2E-16
	Genotype:Training	7	1.20	0.2997	8.32	3.053E-01
	Genotype:Age	7	11.98	<.0001	83.88	2.217E-15
	Training:Age	1	2.48	0.1156	2.48	1.154E-01
	Genotype:Training:Age	7	0.56	0.7905	3.90	7.907E-01
	Residuals	1488				
Unexercised		DF	F-value	p-value	Chisq	Pr(>Chisq)
	(Intercept)	1	11351.95	<.0001		
	Nuclear	1	42.20	<.0001	45.40	1.606E-11
	mtDNA	3	4.03	0.0074	11.09	1.127E-02
	Age	1	1389.44	<.0001	1390.81	<2.2E-16
	Nuclear:mtDNA	3	3.33	0.0193	9.96	1.889E-02
	Nuclear:Age	1	7.90	0.0051	7.76	5.354E-03
	mtDNA:Age	3	6.65	0.0002	19.96	1.730E-04
	Nuclear:mtDNA: Age	3	8.46	<.0001	25.38	1.286E-05
	Residuals	744				
Exercised		DF	F-value	p-value	Chisq	Pr(>Chisq)
	(Intercept)	1	8986.16	<.0001		
	Nuclear	1	47.05	<.0001	50.14	1.435E-12
	mtDNA	3	5.25	0.0014	16.08	1.090E-03

	Age	1	905.04	<.0001	906.61	<2.2E-16
	Nuclear:mtDNA	3	6.74	0.0002	20.44	1.379E-04
	Nuclear:Age	1	11.16	0.0009	11.18	8.281 E-04
	mtDNA:Age	3	4.42	0.0043	13.25	4.119E-03
	Nuclear:mtDNA:Age	3	4.17	0.0061	12.50	5.855E-03
	Residuals	744				
Percent Change (Figure 2D&E)		DF	F-value	Sum Sq	Pr(>F)	
	Nuclear	1	149.20	1439.8	<2.2E-16	
	mtDNA	3	130.93	3790.4	<2.2E-16	
	Nuclear:mtDNA	3	99.53	2881.4	<2.2E-16	
	Residuals	152		1466.8		
Residual standard error: 3.106 on 152 degrees of freedom Multiple R-squared: 0.8469, Adjusted R-squared: 0.8398 F-statistic: 120.1 on 7 and 152 DF, p-value: <2.2e-16						

Table 2

ANOVAs of Endurance by Genotype and Training

Ra/La					
Combined	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Genotype	3	741754	140.68	<2E-16
	Training	1	102800	58.49	2.940E-10
	Genotype:Training	3	106176.00	20.14	5.590E-09
	Residuals	56	98422.00		
Residual standard error: 41.92 on 56 degrees of freedom Multiple R-squared: 0.9062, Adjusted R-squared: 0.8945 F-statistic: 77.28 on 7 and 56 DF, p-value: < 2.2e-16					
	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Nuclear	1	18057	10.2739	0.00233
	mtDNA	1	717197	408.0703	<2.2E-16
	Training	1	102800	58.4913	2.94E-10
	Nuclear:mtDNA	1	6500	3.6986	0.05955
	Nuclear;Training	1	3379	1.9223	0.1711
	mtDNA:Training	1	97266	55.3423	6.05E-10
	Nuclear;mtDNA;Training	1	5532	3.1474	0.08149
	Residuals	98422	56		
Residual standard error: 41.92 on 56 degrees of freedom Multiple R-squared: 0.9062, Adjusted R-squared: 0.8945					
Unexercised	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Nuclear	1	2907	2.408	0.1319
	mtDNA	1	671351	556.0972	<2E-16
	Nuclear:mtDNA	1	20	0.0162	0.8997
	Residuals	28	33803		
Residual standard error: 34.75 on 28 degrees of freedom Multiple R-squared: 0.9523, Adjusted R-squared: 0.9471 F-statistic: 186.2 on 3 and 28 DF, p-value: < 2.2e-16					
Exercised	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Nuclear	1	18528	8.0284	0.008445
	mtDNA	1	143113	62.0122	1.41E-.08
	NuclearmtDNA	1	12013	5.2051	0.030326
	Residuals	28	64619		
Residual standard error: 48.04 on 28 degrees of freedom Multiple R-squared: 0.7288, Adjusted R-squared: 0.6997 F-statistic: 25.08 on 3 and 28 DF, p-value: 4.36e-08					
Mitoswitch					

[a]Single replicate	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Genotype	9	1970323	17.65	<2E-16
	Training	1	122047	9.8396	0.002082
	Genotype:Training	9	341335	3.0577	0.002254
	Residuals	140	1736515		
Residual standard error: 111.4 on 140 degrees of freedom Multiple R-squared: 0.5836, Adjusted R-squared: 0.5271 F-statistic: 10.33 on 19 and 140 DF, p-value: <2.2e-16					
	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Genotype	9	2937788	25.7026	<2E-16
	Training	1	297622	23.4348	2.688E-.06
	Genotype:Training	9	408360	3.5727	0.0004016
	Residuals	188	2387600		
Residual standard error: 112.7 on 188 degrees of freedom Multiple R-squared: 0.6041, Adjusted R-squared: 0.5641 F-statistic: 15.1 on 19 and 188 DF, p-value: <2.2e-16					
[a]Multiple replicate	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Genotype	12	3003960	19.8597	<2.2E-16
	Training	1	297622	23.6071	2.54E-06
	Genotype:Training	12	435446	2.8785	0.001167
	Residuals	182	2294342		
Residual standard error: 112.3 on 182 degrees of freedom Multiple R-squared: 0.6196, Adjusted R-squared: 0.5673 F-statistic: 11.86 on 25 and 182 DF, p-value: <2.2e-16					
Combined	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Genotype	17	1260238	13.2686	2.29E-12
	Training	1	52124	3.8416	0.05248
	Genotype:Training	7	288620	3.0388	0.005787
	Residuals	112	1519660		
Residual standard error: 116.5 on 112 degrees of freedom Multiple R-squared: 0.513, Adjusted R-squared: 0.4478 F-statistic: 7.866 on 15 and 112 DF, p-value: 8.66e-12					
Combined	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Nuclear	1	1110981	81.8801	5.16E-15
	mtDNA	3	30103	0.7395	0.530637
	Training	1	52124	3.8416	0.5248
	Nuclear:mtDNA	3	119154	2.9272	0.036869
	Nuclear;Training	1	245	0.018	0.8934
	mtDNA:Training	3	70392	1.7293	0.165052
	Nuclear;mtDNA;Training	3	217983	5.3552	0.001751

	Residuals	112	1519660		
Residual standard error: 116.5 on 112 degrees of freedom Multiple R-squared: 0.513, Adjusted R-squared: 0.4478 F-statistic: 7.866 on 15 and 112 DF, p-value: 8.66e-12					
Unexercised	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Nuclear	1	539123	48.6235	3.83E-09
	mtDNA	3	80235	2.4121	0.7632
	Nuclear:mtDNA	3	314700	9.4609	3.75E-05
	Residuals	56	620912		
Residual standard error: 105.3 on 56 degrees of freedom Multiple R-squared: 0.6007, Adjusted R-squared: 0.5508 F-statistic: 12.03 on 7 and 56 DF, p-value: 2.952e-09					
Exercised	Term in models	DF	Sum Sq	F-value	Pr(>F)
	Nuclear	1	572103	35.6471	1.70E-07
	mtDNA	3	20260	0.4208	0.7388
	Nuclear:mtDNA	3	22437	0.466	0.7072
	Residuals	56	898748		
Residual standard error: 126.7 on 56 degrees of freedom Multiple R-squared: 0.4062, Adjusted R-squared: 0.332 F-statistic: 5.472 on 7 and 56 DF, p-value: 7.959e-05					

[a]"Single replicate" analysis includes one cohort of 8 vials of each group performed at the same time.

[a]"Multiple replicates" model includes all data from single replicate model plus an additional cohort of 8 vials for OreR, Zim53 and w^{1118} from Figure 3. See methods for detailed description of statistical models.

Table 3

Post-training assessment

Pacing		**Degrees of Freedom**	**Chi-Squared**		**n**	**P-value**
	Nucleotype **Mitotype**	2 4	28.914 50.051		≥149	<0.0001 <0.0001
Unexercised	**Nucleotype** **Mitotype**	2 4	23.799 30.802		≥79	<0.0001 <0.0001
Exercised	**Nucleotype** **Mitotype**	2 4	8.2195 43.839		≥72	<0.0001 0.01641
Flight		**Degrees of Freedom**	**Sum of Squares**	**F-value**	**n**	**Pr(>F)**
	Nuclear	1	202	0.3295	≥149	0.5660228
	mtDNA	3	8028	4.3719		0.0045025
	Training **Nuclear:mtDNA** **Nuclear:Training** **mtDNA;Training** **Nu:mtDNA:Train** **Residuals**	1 3 1 3 3 1495	20947 14604 8534 8348 5976 915024	34.2248 7.9535 13.9434 4.5462 3.2548		6.022E-09 2.913E-05 0.0001955 0.0035343 0.029496
Residuals: Sum of Squares: 915024 Residual standard error: 24.74 on 1495 degrees of freedom Multiple R-squared: 0.06828, Adjusted R-squared: 0.05893 F-statistic: 7.304 on 15 and 1495 DF, p-value: 8.681e-16						
Unexercised		**Degrees of Freedom**	**Sum of Squares**	**F-value**	**N**	**Pr(>F)**
	Nucleotype	1	3425	7.1972	≥78	0.007478
	Mitotype **Interaction**	3 3	6821 2712	4.7787 1.9001		0.002647 0.12208
Residuals: Sum of Squares: 325936 Residual standard error: 21.81 on 685 degrees of freedom Multiple R-squared: 0.03759, Adjusted R-squared: 0.02775 F-statistic: 3.822 on 7 and 685 DF, p-value: 0.0004379						
Exercised		**Degrees of Freedom**	**Sum of Squares**	**F-value**	**N**	**Pr(>F)**
	Nucleotype **Mitotype** **Interaction**	1 3 3	5389 9529 17868	7.4103 4.3676 8.1895	≥71	0.006624 0.004624 2.249E-05
Residuals: Sum of Squares: 589088 Residual standard error: 26.97 on 810 degrees of freedom Multiple R-squared: 0.05226, Adjusted R-squared: 0.04407 F-statistic: 6.38 on 7 and 810 DF, p-value: 2.546e-07						
Lysotracker		**Degrees of Freedom**	**Sum of Squares**	**F-value**	**n**	**Pr(>F)**
	Genotype	9	937.19	9.5159	20	8.210E-12
	Training	1	395.15	36.1096		1.011E-08
	Interaction	9	1660.08	16.8558		<2.2E-16
Residuals: Sum of Squares: 1969.73 Residual standard error: 3.308 on 180 degrees of freedom Multiple R-squared: 0.603, Adjusted R-squared: 0.5611 F-statistic: 14.39 on 19 and 180 DF, p-value: <2.2e-16						
Unexercised		**Degrees of Freedom**	**Sum of Squares**	**F-value**	**n**	**Pr(>F)**

	Nucleotype	1	44.88	5.3977	10	0.02299
	Mitotype	3	601.99	24.1345		6.457E-11
	Interaction	3	231.09	9.2646		2.939E-05
Residuals: Sum of Squares: 1718.27 Residual standard error: 3.454 on 144 degrees of freedom Multiple R-squared: 0.5799, Adjusted R-squared: 0.5361 F-statistic: 13.25 on 15 and 144 DF, p-value: <2.2e-16						
Exercised		**Degrees of Freedom**	**Sum of Squares**	**F-value**	**n**	**Pr(>F)**
	Nucleotype	1	62.37	4.0108	10	0.0489765
	Mitotype	3	342.72	7.3464		0.0002306
	Interaction	3	870.84	18.6667		4.649E-09
Residuals: Sum of Squares: 598.63 Residual standard error: 2.883 on 72 degrees of freedom Multiple R-squared: 0.5946, Adjusted R-squared: 0.5552 F-statistic: 15.09 on 7 and 72 DF, p-value: 5.763e-12						