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Exercising *D. melanogaster* modulates the mitochondrial proteome and physiology - the effect on lifespan depends upon age and sex.

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Abstract:

Ageing is a major risk factor for many of the most prevalent diseases, including neurodegenerative disease, cancer and heart disease. As the global population continues to age, behavioural interventions that can promote healthy ageing will improve quality of life and relieve the socio-economic burden that comes with an aged society. Exercise is recognised as an effective intervention against many diseases of ageing, but we don't know the stage in an individual's lifetime in which exercise is most effective at promoting healthy ageing and whether it has a direct effect on lifespan. We exercised w^{1118} *Drosophila melanogaster,* interrogating effects of sex and group size, at different stages of their lifetime and recorded their lifespan. Climbing scores at 30 days were measured to record differences in fitness in response to exercise. We also assessed the mitochondrial proteome of w^{1118} *Drosophila* that had been exercised for one week, alongside mitochondrial respiration measured using High-Resolution Respirometry, to determine changes in mitochondrial physiology in response to exercise. We found that age-targeted exercise interventions improve lifespan in male and female *Drosophila*, and grouped males exercised in late life had improved climbing scores, when compared with those exercised throughout their entire lifespan. The proteins of the electron transport chain were significantly upregulated in expression after one week of exercise, and complex II linked respiration was significantly increased in exercised *Drosophila*. Taken together our study provides a basis to test specific proteins and complex II of the respiratory chain as important effectors of exercise induced healthy ageing.

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Introduction

The dramatic ageing of the global population is a well-documented phenomenon. The World Health Organisation estimates that there are currently over 900 million people over the age of 60, and by 2050 this is set to increase to 2 billion[1]. With this demographic transformation, there will be huge economic costs incurred due to the health and social care needs of this group, in particular with the increased occurrence of non-communicable diseases. Within the EU it has been estimated that over 65s already account for over 40% of healthcare spending, a figure that will increase as this demographic continues to expand[2]. It is therefore paramount that we develop a comprehensive understanding of the biology of ageing and how we can increase the healthspan of individuals as lifespan continues to rise.

Lifespan can be defined as the length of time between the birth and death of an individual, however the definition of healthspan is different. One common definition of healthspan is '*healthspan is the period of life spent in good health, free from the chronic diseases and disabilities of ageing*', the potential pitfalls of this definition are clear[3]. In humans, females live longer average lifespans in populations with both shorter and longer life expectancies[4]. Further, lifestyle considerations can also influence the outcome of lifespan and healthspan, and these can include whether an individual lives a solitary or social lifestyle. When considering the sociality of animals and lifespan, those that are obligately social tend to have greater lifespan when they have more associates and stronger social bonds with those associates[5–8], however this benefit is not universal, and in particular there are differences for species with facultative sociality[9–11].

The biology of ageing itself is a complex picture but has characteristic hallmarks: genomic instability, telomere attrition, epigenetic alterations, loss of proteostasis, deregulated nutrient sensing, mitochondrial dysfunction, cellular senescence, stem cell exhaustion, and altered intercellular communication[12]. Due to the central role that functional mitochondria play in metabolism and intracellular signalling[13–17], the regulation of apoptosis[18] and their well-documented dysfunction in a broad range of age-related pathologies[19–21], a fundamental understanding of their role in the ageing process will anchor developments in the field.

Exercise is recognised as being pivotal in the fight to keep individuals healthier for longer. While exercise has long being advocated for as a broad-spectrum remedy against ill-health, many of the molecular details that underpin this have remained elusive. However, as the weight of evidence for exercise as a protective strategy accumulates, our comprehension of how exercise extends lifespan is increased[22]. These molecular changes are now known to include exercise-induced oxidative stress, in line with the mitohormesis[23,24].

Drosophila are a viable model for both ageing and lifespan studies as well as mitochondrial studies[25,26]. This is due to their well understood genetics, the high proportion of homologous genes they possess with *H. sapiens* including those implicated in disease, as well as their relative ease in husbandry. Examples of lifespan assays in *Drosophila* include their use to explore the effect of D-GADD45, Cu/ZnSOD and MnSOD overexpression and survival outcomes[27,28]. However, lifespan assays have more traditionally been used in human and mammalian studies. Landmark mitochondrial studies in *Drosophila* were those which presented mechanistic evidence of Pink1 and Parkin dysfunction in Parkinson's disease[29–31].

Proteomics is the study of the entire complement of proteins present within a biological sample [32,33]. In this instance, we focussed upon the proteome of the mitochondrion, which facilitates a deep evaluation of the physiology of the mitochondrion in both health and disease.

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While it is recognised that exercise is an effective means of delaying the onset of certain hallmarks of ageing, and in preventing the onset of disease of ageing, there is less certainty around how exercise should be applied as an intervention and which molecular mechanisms are modulated to influence the ageing process. In this study, we explored exercise interventions at different stages of life for *Drosophila* and recorded how this affected their survival probabilities, plotting Kaplan-Meier survival curves with log-rank test analysis. To assess sex differences and the effects of a grouped or solitary lifestyle, we exercised flies that were housed singly females and males, also in groups of females and males. Climbing assay scores at 30 days were also measured to assess age-associated fitness correlated with exercise. To understand the molecular changes that are associated with differences in survival outcomes, we assessed the mitochondrial proteome via 2DE-MS and label-free mass spectrometry of *Drosophila* that had been exercised for one week. We measured mitochondrial fitness by high resolution respirometry to specifically reveal mitochondrial responses to exercise.

Methods:

Fly Husbandry

We used *Drosophila melanogaster* strain w^{1118}.

Fly food (Quick Mix Medium, Blades Biological) was added to the vial, to a depth of 1cm and 3ml of distilled water was added, it was left for one minute and then a small sprinkle of yeast was added. The singly housed *Drosophila* from further trials were given the same amount of food as those kept in groups. *Drosophila* were transferred to new food once a week on average and were kept in an incubator at 25°C. Food was kept hydrated with 150 µl of distilled water every day. When food became dry, or the flies laid eggs they were moved to a new vial and transferred back following food rehydration. The light cycle varied depending when lights were turned off/on in the laboratory but was generally a 12-hour cycle.

Fly Separation

Flies in glass vials were cooled on ice for 5-10 minutes, they were placed under a microscope to determine sex and then placed into vials accordingly. The groups were 20 flies in each vial. These were labelled GF/GM for exercised group females/males. Ten individual flies of known sex were placed in separate vials, labelled SF/SM for single females/males. Two cohorts of flies were exercised in independent experiments, and the data sets for the two cohorts were then pooled for the statistical analysis of the lifespan assay.

Exercise Regime

The methodology for the exercise regime was modified from the protocol used by Tinkerhess *et al*[34]. The exercise machine utilised a *power tower* strategy that taps the flies down every 15 seconds to induce a negative geotaxis behaviour. The flies were exercised within a 25°C incubator. The flies were transferred to empty vials during the exercise sessions. Flies were exercised at 10.30am for ten minutes on Monday, Wednesday and Friday, two-day rest on Saturday and Sunday.

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For lifespan assay, flies were subjected to one of the following five exercise regimes: lifetime exercise (weeks 1 – 6 of life), early life exercise (weeks 1 – 2 of life), middle life exercise (weeks 3 – 4 of life), late life exercise (weeks 5 – 6 of life), or no exercise.

For proteomic analysis mixed sex flies that were 1 – 4 days post-eclosion were placed into vials of 20 flies and exercised for one week before being sacrificed by freezing at -80°C, one hour after the final exercise period.

Mortality

Deaths were recorded throughout the experiment. Mortality was analysed using GraphPad Prism 8 by inputting data into Kaplan-Meier survival graphs. Log-rank test ($P<0.05$) was carried out between groups of interest.

Climbing Assay

A modified *power tower* protocol was used to perform a 'RING assay'[35]. Flies were moved into empty vials and images were taken 4 seconds after the frame had been tapped down. A climbing index score was calculated by multiplying the number of flies per quadrant score and dividing by the number of flies in the vial. Movie player classic was used to create frames to analyse, edited in Microsoft Paint to add lines and distinguish the 4 quadrants. A mean was calculated (n=6), and unpaired t test performed between means of different groups 30 days into their lifespan, using GraphPad Prism.

Tissue preparation for HRR

High-resolution respirometry (HRR) analysis was carried out on the sixth day after the start of the exercise or control treatment. To prepare the tissue, flies were cooled on ice before five were randomly chosen and mechanically homogenised in 500µl of MiR05 buffer (Oroboros Instruments - 0.5mM EGTA, 3mM $MgCl_2$, 60mM Lactobionic acid, 20mM Taurine, 10mM KH_2PO_4, 20mM HEPES, 110mM D-Sucrose, 1g/L BSA, pH 7.1). The homogenate was briefly spun and only the supernatant used in order to exclude non-cellular debris. The sample was kept on ice until HRR analysis.

High-resolution respirometry

HRR was carried out using the Oroboros Oxygraph-2k (Oroboros® Instruments, Innsbruck, Austria). The electrodes were calibrated daily to ensure oxygen consumption was consistent and analysis was carried out at 20°C. 100ul of the fly homogenate was added to each chamber before the following substrates were added: i) 5µl and 10µl of the complex I

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substrates, pyruvate and malate (5mM and 2mM, respectively), ii) 20µl of 10mM succinate, a complex II substrate, iii) 1µl titrations of 0.5µM CCCP, and finally iv) 1µl of 2.5µM antimycin A, a complex III inhibitor.

Mitochondrial preparation

Flies were placed in 250 µL of mitochondrial isolation buffer, then subjected to 5 mins of manual homogenisation with a 1.2 – 2.0 mL Eppendorf micropestle. Fractions were obtained using protocols described previously [36].

2D gel electrophoresis (2D – PAGE)

DM mitochondrial fractions and whole-fly homogenates were subject to isoelectric focussing using the ZOOM IPG system and pH 3 – 10 (non-linear) ZOOM IPG strips following the manufacturers protocol (Life Technologies). Gels were stained and imaged before excision of protein spots prior to identification. Analyses were performed using SameSpots software (one-way Anova).

Matrix-assisted laser desorption ionization tandem time-of-flight mass spectrometry (MALDI – TOF/MS)

Samples were analysed at the Centre of Excellence in Mass Spectrometry at the University of York. Proteins were reduced and alkylated, followed by digestion in-gel with trypsin. MALDI-TOF/MS was sued to analyse the samples. The generated tandem MS data were compared against the NCBI database using the MASCOT search programme to identify the proteins. *De novo* sequence interpretation for individual peptides was inferred from peptide matches.

Liquid chromatography tandem mass spectrometry (LC – MS/MS)

Mitochondrial fractions were analysed by the Centre of Excellence in Mass Spectrometry at the University of York. Proteins were reduced and alkylated, followed by digestion in-gel with trypsin. LC-MS/MS was used to analyse the samples. Resulting LC-MS/MS data were imported in PEAKS StudioX for peak picking, peptide identification and precursor-intensity based relative protein quantification. Extracted tandem MS data were searched against the combined *D. melanogaster* and *S. cerevisiae* subsets of the UniProt database. Protein

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identifications were filtered to achieve <1% false discovery rate (FDR), and to require a minimum of two unique peptides per protein group.

For relative label-free quantification, extracted ion chromatograms for identified peptides were extracted and integrated for all samples. Protein abundances were normalised between samples on the basis of total identified peptide ions area. Significance was established using the PEAKSX interpretation of the significance B model. The multiple test corrected FDR thresholds. A *-log10p value* >23.52 (1% FDR) was deemed significant using the model.

Bioinformatic analyses

To state that a protein had a significant abundance changes between the exercised and non-exercised groups we developed the following criteria: the protein must be quantified in all three biological replicates, the relative abundance ratios (RAR [exercise/non-exercise]) had to be either <0.67 or >1.5 and have a *-log10p value* >23.52. Proteins that were not quantified in more than one biological triplicate in only one of the treatment groups were also noted of interest.

Two representative heatmaps were developed: one for proteins with increased abundance (RAR >1.5) and one for proteins with decreased abundance (RAR <0.67) post-exercise. All three biological replicates for each group were included with relative abundances depicted using a red-to-blue (high-to-low relative abundance) colour gradient. Proteins were further analysed using the STRING database v.11.0 to examine functional relationships between proteins significantly different in abundance between exercised and non-exercised groups. The platform was used to create two protein-protein interaction (PPI) networks. To narrow down proteins of interests, those with no associated interactions in the network were hidden and protein nodes were coloured based on their biological process, molecular function and cellular component designation as per Gene Ontology (GO) and UniProt annotation.

Results:

In males late-life exercise has the most beneficial effect and exercise throughout life is detrimental in comparison.

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Male Drosophila exercised throughout their lifetime have worse survival outcomes when compared with groups of males exercised at any other age or not exercised at all.

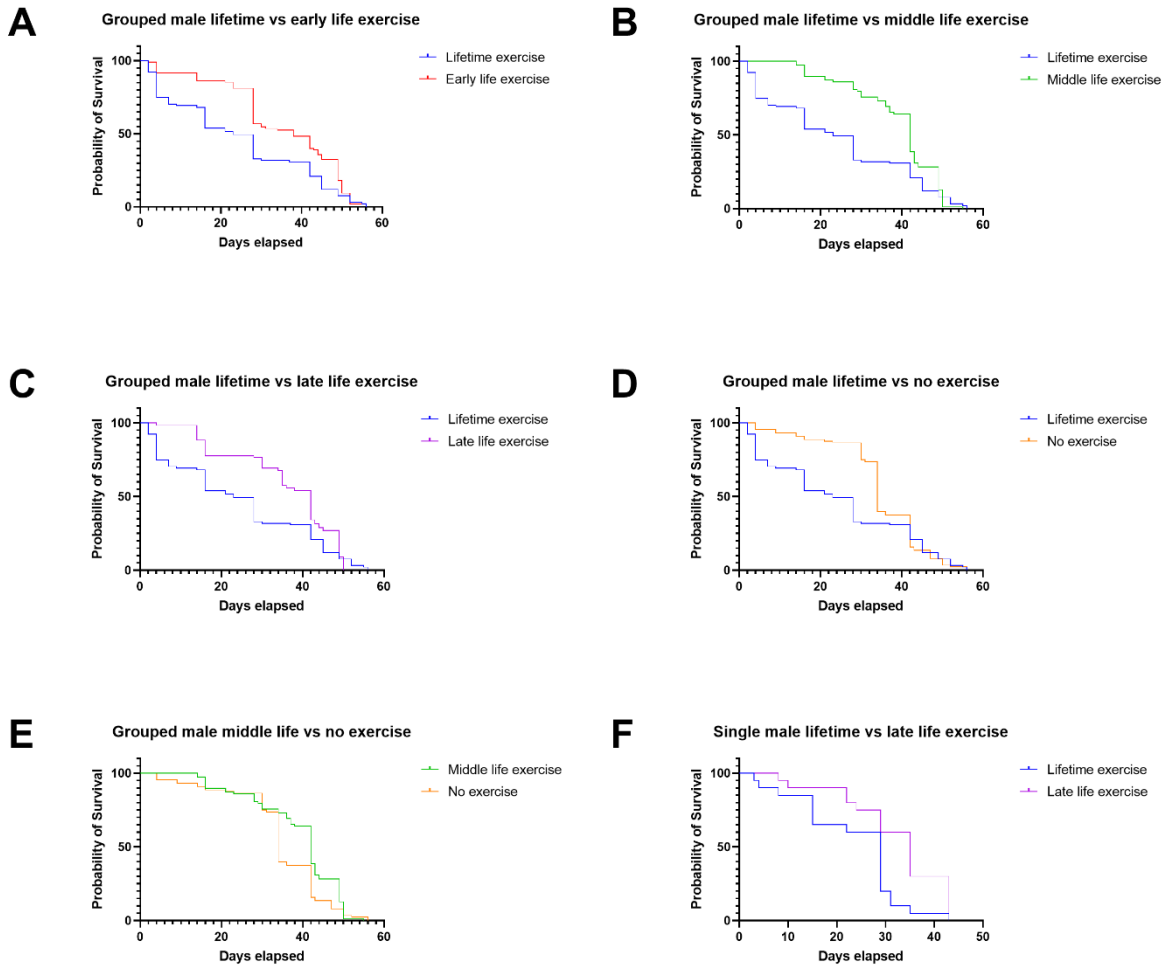

Figure 1 - Percentage probability of survival for grouped and single male flies. A) Grouped male flies lifetime exercise vs early life exercise (N=93, 95)(Log-rank test p=0.0011), B) Grouped male lifetime vs middle life exercise (N=93, 78)(Log-rank test p=0.0014), C) Grouped male lifetime vs late life exercise (N=93, 121)(Log-rank test p=0.0050), D) Grouped male lifetime vs no exercise (N=93, 87)(Log-rank test p=0.0237), E) Grouped male middle life vs no exercise (N=78, 87)(Log-rank test p=0.0125), F) Single male lifetime vs late life exercise (N=20, 20)(Log-rank test p=0.0053).

Grouped male *Drosophila* that were exercised throughout their lifetime had a worse percentage probability of survival than those that were exercised in early life, middle life, and late life, as well as those that were not exercised at all (**Figure 1A-D**). Grouped male *Drosophila* that were exercised in middle life had improved percentage probability of survival compared with those that were not exercised at all (**Figure 1E**). Only single male *Drosophila* that were exercised during late life had improved percentage survival probability compared with those exercised throughout their life (**Figure 1F**), which was also observed in the grouped male flies.

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In groups of female Drosophila age-targeted exercise is more beneficial than lifetime exercise, late life exercise extends lifespan in singly housed female individuals.

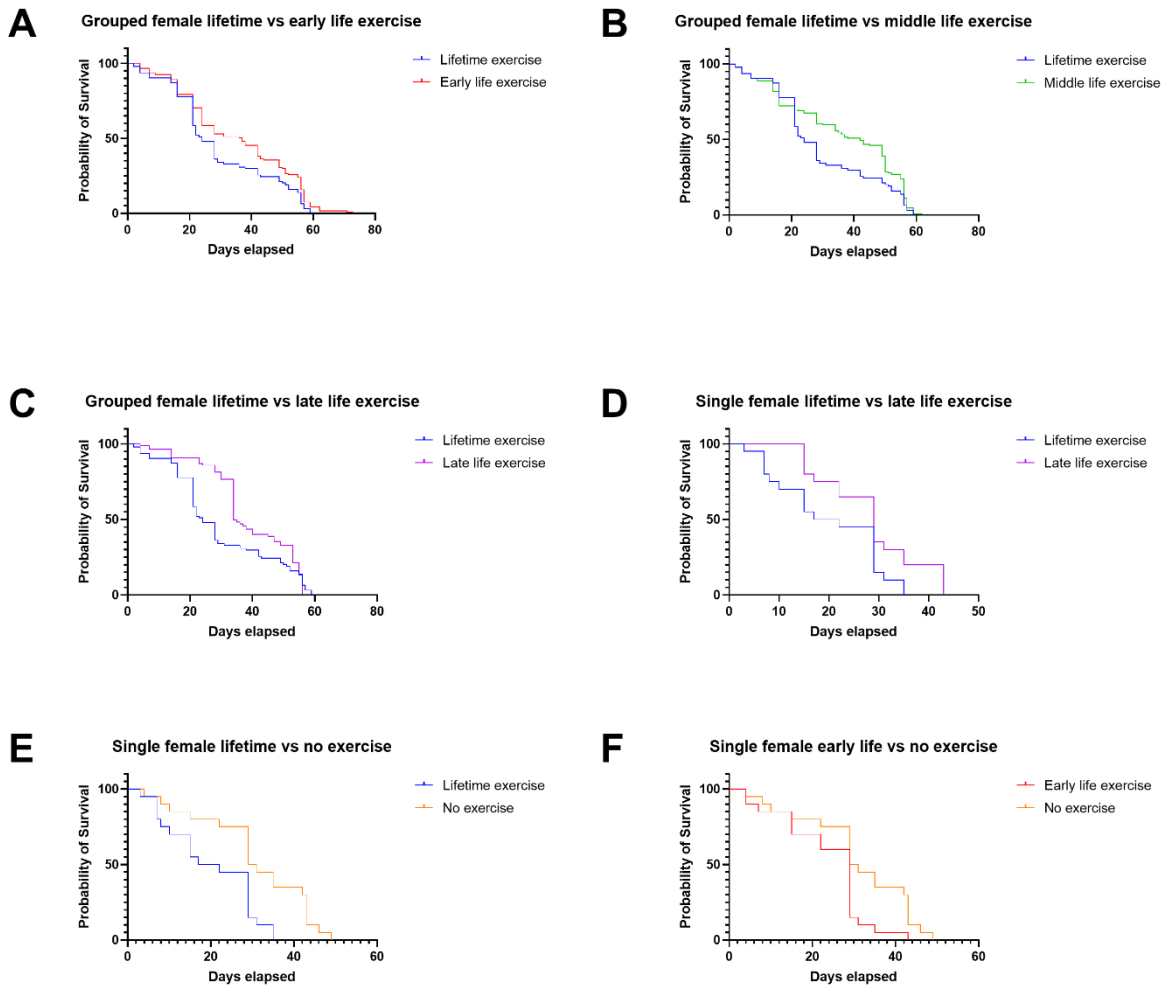

Figure 2 – Percentage probability of survival for grouped and single female flies. A) Grouped female lifetime vs early life exercise (N=94, 121)(Log-rank test p=0.0094), B) Grouped female lifetime vs middle life exercise (N=94, 127)(Log-rank test p=0.0141), C) Grouped female lifetime vs late life exercise (N=94, 85)(Log-rank test p=0.0241), D) Single female lifetime vs late life exercise (N=20, 20)(Log-rank test p=0.0202), E) Single female lifetime vs no exercise (N=20, 20)(Log-rank test p=0.0025), F) Single female early life vs no exercise (N=20, 20)(Log-rank test p=0.0202).

Grouped female *Drosophila* exercised in early, middle and late life had increased percentage probability of survival compared with those that were exercised throughout their life (**Figure 2A-C**). As observed in male *Drosophila*, the single females that were exercised in late life also had improved percentage probability of survival compared with those that were exercised throughout their life (**Figure 2D**). Single females that were not exercised at all also had increased percentage probability of survival compared with flies that were exercised throughout their lifetime (**Figure 2E**), as well as those exercised in early life (**Figure 2F**).

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Figure 3 - Grouped male Drosophila under different exercise regimens produced different climbing scores when compared with lifetime exercise. A) Lifetime exercise vs middle life exercise (N=6) (unpaired t test, p=0.0051), B) Lifetime exercise vs late life exercise (N=6) (unpaired t test, p=0.0183), C) Lifetime exercise vs no exercise (N=6) (unpaired t test, p=0.0344).

When the climbing scores at 30 days were assessed for the *Drosophila* lifespan assay data in Figures 1 and 2, we found that there were significant differences in climbing score outcomes (**Figure 3**). Flies that were exercised in the middle of their life, and flies not exercised at all, had lower climbing scores than flies that been exercised throughout their lifetime. However, flies exercised in late-life had an improved climbing score compared with those exercised throughout their lifetime, correlating with the improved lifespan assay outcome.

High Resolution Respirometry of exercised *Drosophila*

We found a significant difference in the mean flux control ratio (FCR) of exercised and non-exercised flies when succinate, a complex II substrate, was added after the addition of pyruvate and malate (0.32 and 0.13, respectively; student's t-test p-value = 0.025). Furthermore, the mean electron transport (ET) capacity was significantly greater in non-exercised flies compared to the exercised flies (42.55 and 14.27; student's t-test p-value = 0.002) (**Figure 4**, **Table 1**).

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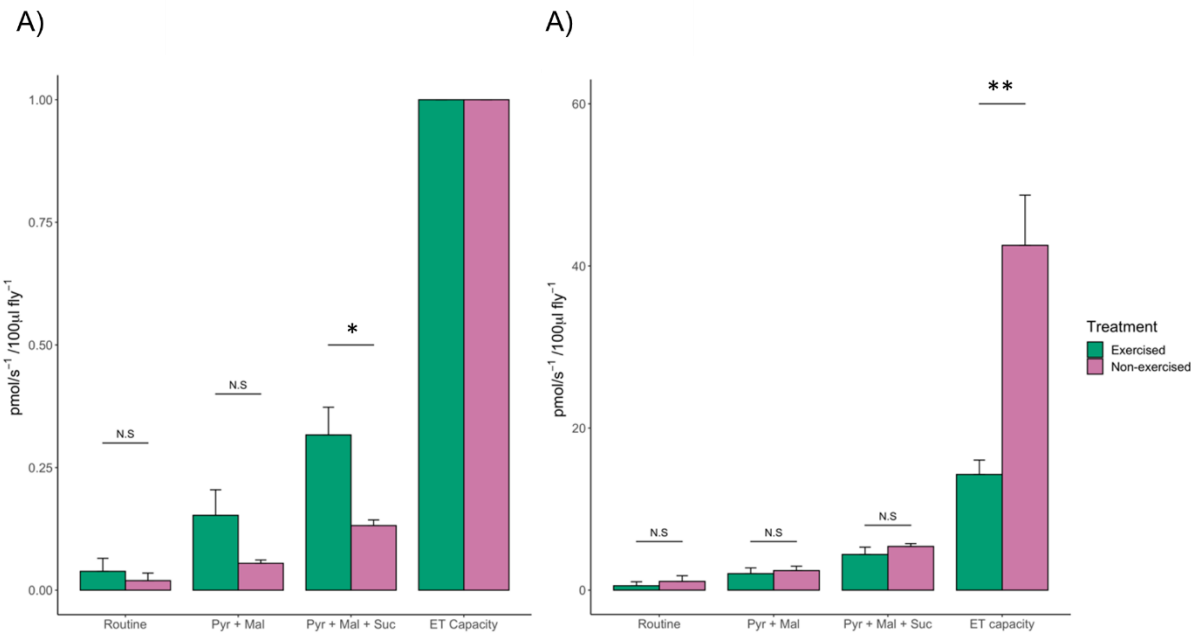

Figure 4 - A) specific flux oxygen consumption; and B) flux control ratio (FCR), in exercised and non-exercised male D. melanogaster. Significant p-values: A) succinate p-value = 0.025; B) ET capacity p-value = 0.002.

The spare respiratory capacity (SRC) is calculated as a relative value by (ET capacity specific flux) / (Routine specific flux * 100), where the SRC for non-exercised and exercised flies is 0.39 and 0.26, respectively.

Table 1 - Mean values of the specific flux and flux control ratio in exercised and non-exercised male D. melanogaster. p-values from unpaired students t-test.

Stage		Exercised n= 5 Mean(s.e)	Non-exercised n = 4 Mean(s.e)	*p*-value
Routine				
	Specific flux	0.55(0.48)	1.08(0.70)	0.537
	FCR	0.04(0.03)	0.02(0.02)	0.58
Pyruvate & Malate				
	Specific flux	2.04(0.70)	2.41(0.54)	0.699
	FCR	0.15(0.05)	0.05(0.01)	0.143
Succinate				
	Specific flux	4.40(0.89)	5.39(0.35)	0.381
	FCR	0.32(0.06)	0.13(0.01)	**0.025***
ET capacity				
	Specific flux	14.27(1.77)	42.55(6.19)	**0.002****

significant differences in protein expression (one-way ANOVA) were determined using SameSpots (Totallab). Protein identification was conducted using 2D-PAGE followed by MALDI-TOF/MS. Data are the average of three biological replicates.

Spot no.	Protein identity	Anova (p)	Fold Change	Exercise related change
519	Arginine kinase OS=Drosophila melanogaster	0.003	2	Reduced
784	NADH-ubiquinone oxidoreductase 75 kDa subunit	0.004	1.5	Increased
773	Alcohol dehydrogenase	0.012	1.7	Reduced
791	Actin, larval muscle	0.024	2.7	Reduced
788	IP09655p (malate dehydrogenase) Succinate-CoA ligase [ADP/GDP-forming] subunit alpha ATP synthase subunit alpha	0.037	1.8	Reduced

Protein changes after one week of exercise.

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2DE-MS analysis of mitochondrial fractions from exercised and non-exercised male flies indicated six proteins with reduced levels of expression and Complex I subunit NASH-ubiquinone oxidoreductase 75 kDa subunit had increased expression (**Table 2**). The proteins with reduced protein expression in response to exercise were identified to be arginine kinase, alcohol dehydrogenase, actin, larval muscle, malate dehydrogenase, succinate-CoA ligase [ADP/GDP-forming] subunit alpha and ATP synthase subunit alpha.

Table 3 – Difference in whole fly protein expression in response to one week of exercise. Statistically significant differences in protein expression (one-way ANOVA) were analysed using SameSpots, Totallab. Protein identification by MALDI-TOF/MS. Data are the average of three biological replicates. F/M/ge, fe/male group exercised; f/m/gne, fe/male group non-exercised.

Group	Spot no.	Protein Identity	Anova (p)	Fold change	Increased in
Fge vs Fse	349	Glycerol-3-phosphate dehydrogenase [NAD(+)], plus a single peptide match to Probable isocitrate dehydrogenase [NAD]	0.008	1.5	Fse
	358	Aconitate hydratase	0.016	1.2	Fge
Fge vs Fgne	351	Glycerol-3-phosphate dehydrogenase [NAD(+)]	0.032	1.7	Fgne
	363	Aconitate hydratase	0.047	1.5	Fge
	375	Vitellogenin-3	0.039	1.6	Fge
Mgne vs Fgne	472	Pyruvate dehydrogenase E1 component subunit beta	0.001	2.2	Mgne
	486	Fructose-bisphosphate aldolase	0.0009479	2.4	Fgne
Mge vs Mgne	487	Dihydrolipoyl dehydrogenase	0.0005776	1.7	Mgne

When comparing the effect of exercise on whole fly protein expression, 2DE-MS analysis revealed that differences in protein levels vary according to the group size and sex of the flies being exercised (**Table 3**). Exercised single females had higher levels of glycerol-3-phosphate dehydrogenase than their grouped female counterparts, while grouped females had higher expression of aconitase hydratase than single females. When considering only grouped females, those that were exercised had increased expression of aconitate hydratase and vitellogenin-3, while their non-exercised counterparts had increased expression of glycerol-3-phosphate dehydrogenase.

2DE-MS detected only one protein with a significant expression change between exercised and non-exercised grouped male *Drosophila*, the non-exercised flies had higher levels of dihydrolipoyl dehydrogenase. When considering sex differences and protein expression, non-exercised grouped males had higher levels of pyruvate dehydrogenase E1 component subunit beta, while non-exercised grouped females had higher levels of fructose bisphosphate aldolase.

There is increased expression of many proteins in response to exercise, including those of the electron transport chain.

2DE-MS was used as a scoping technique to determine proteins of interest based upon changes of their expression in response to exercise, as well as considering the effects of group size and sex of the flies. Label-free mass spectrometry was then used to determine differences in the expression of mitochondrial proteins after *Drosophila* had been exercised

Figure 5 - Representative heatmap of mitochondrial proteins with increased abundance (RAR of >1.5) after 1 week of exercise in D. melanogaster. Red-to-blue colour gradient represents high-to-low relative protein abundance. Proteomic approach used was label-free mass spectrometry. All proteins listed were classified as significant as per criteria described in the results.

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for one week. A total of 515 proteins were identified and quantified in relation to the whole sample.

Of the 515 proteins identified, 424 had significant differences in abundance between the groups that were analysed (>23.52 -log10P, >1% FDR). A total of 337 proteins were quantified in all three replicates of the exercised and non-exercised fly groups. It should be noted that one biological replicate from the exercised *Drosophila* group had less total protein than the other samples, but normalisation procedures compensate for this, and there was no notable effect on the results. Of the 337 commonly identified proteins, 51 had increased expression (RAR>1.5) in response to exercise (**Figure 5**).

STRING database v.11.0 was used to observe protein-protein interactions (PPI) between the 51 proteins with increased expression in response to exercise (**Figure 6**). Of the 51 proteins upregulated in response, 48 were identified by STRING database v.11.0. The PPI enrichment P-value was <1.0e-16, indicating a clear functional enrichment between the proteins with increased abundance in response to exercise.

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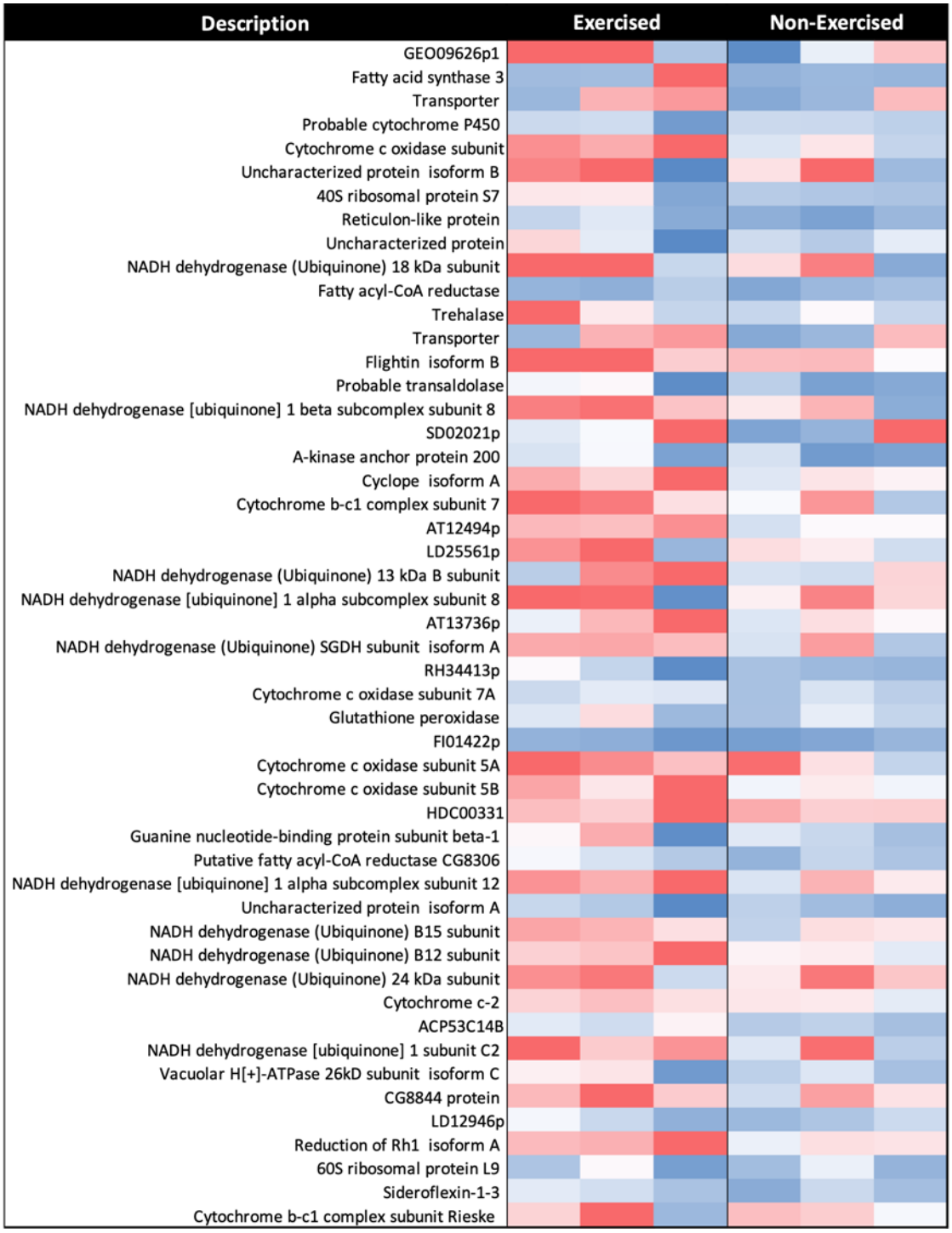

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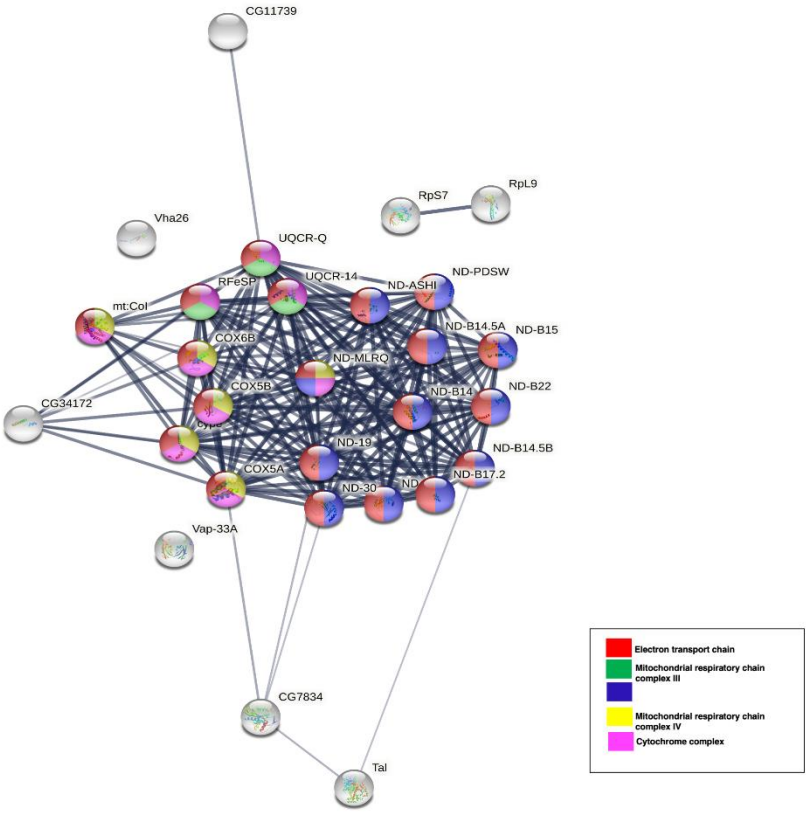

Figure 6 - PPI network of mitochondrial proteins with increased abundance (RAR > 1.5) in response to one week of exercise in D. melanogaster. Network analysis by the STRING v11.0. software, with functional enrichment of (P < 1.0e-16).

In the network presented, no clustering was applied and disconnected nodes in the network are hidden, while line thickness is representative of the strength of the data support. Red nodes represent electron transport chain proteins, blue nodes represent Complex I associated proteins, green nodes represent Complex III associated proteins, yellow nodes represent Complex IV associated proteins, and pink nodes represent cytochrome complex proteins.

Using UniProt and Gene Ontology (GO) annotation for cellular component assignment, we found 27 of the 51 proteins with increased expression in response to exercise localise to the mitochondrion. The other proteins were from either the cytoplasm or other membrane bound organelles, with likely close association with mitochondria resulting in their presence in the mitochondrial isolates.

Of the 27 mitochondrial proteins identified by GO:CC enrichment analysis, 24 are associated with the electron transport chain; 10 from Complex I, 3 from Complex III, 6 from Complex IV and also Cytochrome c-2 (**Table 4**).

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Table 4 – Electron transport chain proteins increased in abundance in response to one week of exercise in D. melanogaster. Enrichment analysis used Gene Ontology Cellular Component allocation from STRING database v.11.0. RAR, relative abundance ratio; FC, fold change.

Description		Accession	Significance (-log10P)	RAR	Log2 FC
Complex I					
NADH dehydrogenase (ubiquinone)	1Subunit C2	Q9VQM2	200	1.53	0.61
	1 Beta subcomplex subunit 8 mitochondrial	Q9W3X7	133.11	1.85	0.89
	1 Alpha subcomplex subunit 8	Q9W125	142.39	1.68	0.74
	1 Alpha subcomplex subunit 12	Q9VQD7	200	1.57	0.65
	SGDH subunit isoform A	Q9VTU2	86.17	1.64	0.71
	B15 subunit	Q6IDF5	36.57	1.56	0.64
	B12 subunit isoform A	Q9W2E8	88.95	1.55	0.64
	24 kDa subunit isoform A	Q9VX36	65.53	1.54	0.63
	18 kDa subunit	Q9VWI0	200	2.05	1.04
	13 kDa Bsubunit	Q9VTB4	78.35	1.69	0.76
GEO09626p1		Q8SYJ2	26.03	2.92	1.55
EG:152A3.7 protein		O97418	29.75	1.9	0.93
CG8844 protein		Q9VQR2	200	1.53	0.61
AT12494p		Q9VJZ4	70.67	1.7	0.77
Complex III					
Cytochrome b-c1 complex	Subunit Rieske mitochondrial	Q9VQ29	80.35	1.5	0.59
	Subunit 7	Q9VXI6	200	1.73	0.79
AT13736p		Q9VVH5	129.49	1.65	0.72
Complex IV					
Cyclope isoform A		Q9VMS1	200	1.78	0.83
Cytochrome C oxidase	Cytochrome C oxidase subunit	Q8IQW2	200	2.14	1.1
	Subunit 7A mitochondrial	Q9VHS2	82.59	1.61	0.69
	Subunit 5A mitochondrial	Q94514	110.61	1.6	0.68
	Subunit 5B isoform A	Q9VMB9	140.51	1.59	0.67
GEO09626p1		Q8SYJ2	26.03	2.92	1.55
Other OXPHOS proteins					
HDC00331		Q6IHY5	200	1.58	0.66
Uncharacterized protein isoform A		Q0KHZ6	93.18	1.56	0.64
Cytochrome c-2		P84029	38.14	1.54	0.62

A subset of mitochondrial proteins decreased in response to one week of exercise in D. melanogaster.

Of the 337 commonly identified proteins from the mitochondrial fraction, across all replicates from the exercise and non-exercise fly groups, there were 36 proteins that had significantly decreased expression in response to exercise (RAR<0.67) (**Figure 7**).

The 36 proteins that were identified as having decreased expression in response to exercise were identified in the STRING v.11.0. database. The PPI enrichment P-value was < 2.96e-08, which indicates functional enrichment between the differentially expressed proteins identified by STRING. Of the 36 proteins, only 11 were identified as localised to the mitochondrion, however many of the non-mitochondrial proteins with differential expression

Figure 7 – Representative heatmap of mitochondrial proteins with decreased abundance (RAR < 0.67) in response to one week of exercise in D. melanogaster. Red-to-blue colour gradient represents high-to-low relative protein abundance. Proteomic analysis was conducted using label-free mass spectrometry. All proteins listed were classified as significant as per criteria described in the results.

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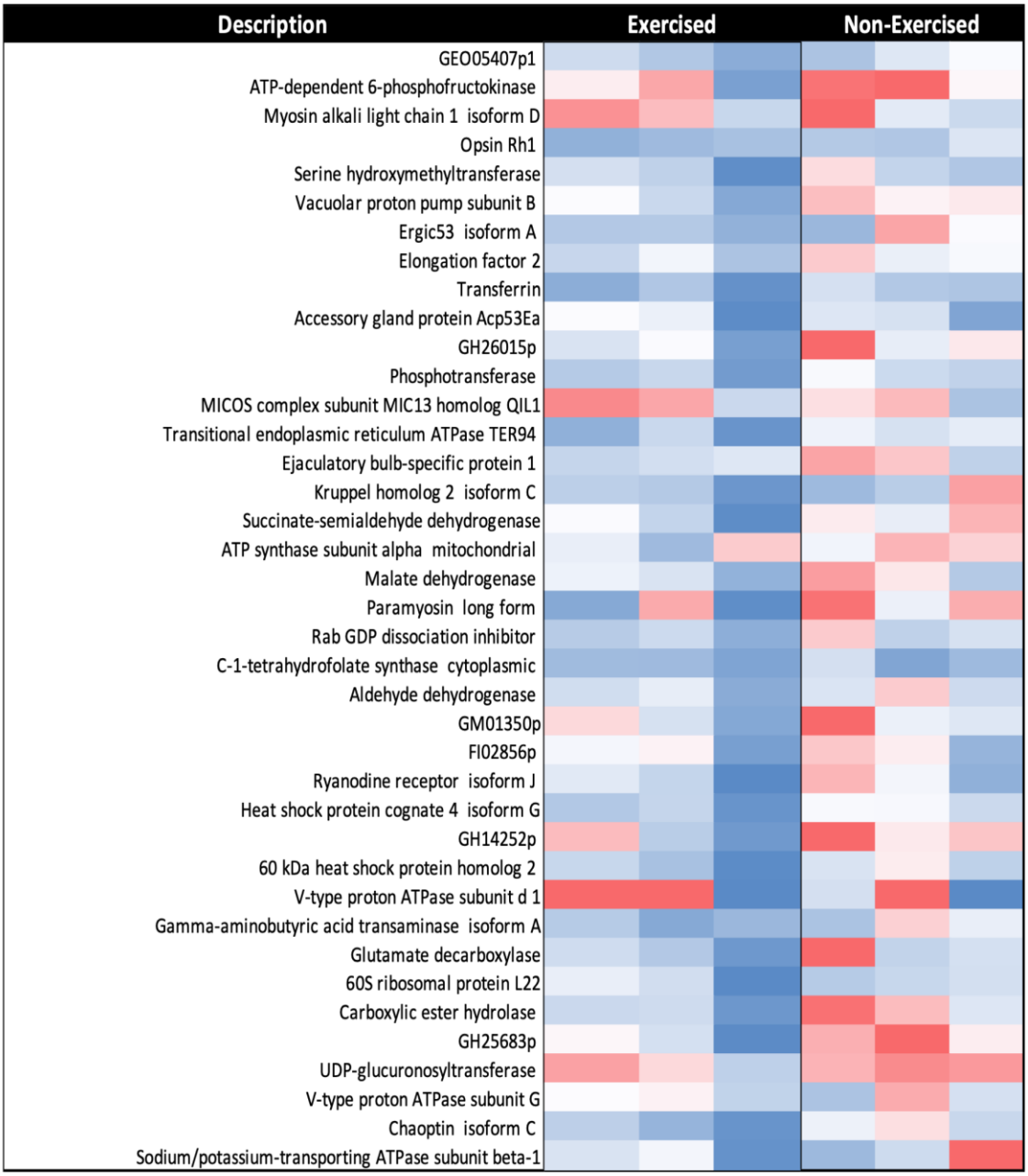

present strong evidence of interaction with the STRING identified mitochondrial proteins (**Figure 8**).

In the PPI network presented clustering was not applied and disconnected nodes were hidden. Red nodes represent proteins in ATP metabolism, light green nodes represent pyruvate metabolism, dark green nodes represent proteins associated with chaperone mediated protein folding, pink nodes represent proteins in carboxylic acid metabolism, dark blue nodes represent proteins in the TCA cycle, and yellow nodes represent proteins associated with cellular amino acid metabolism (**Figure 8**).

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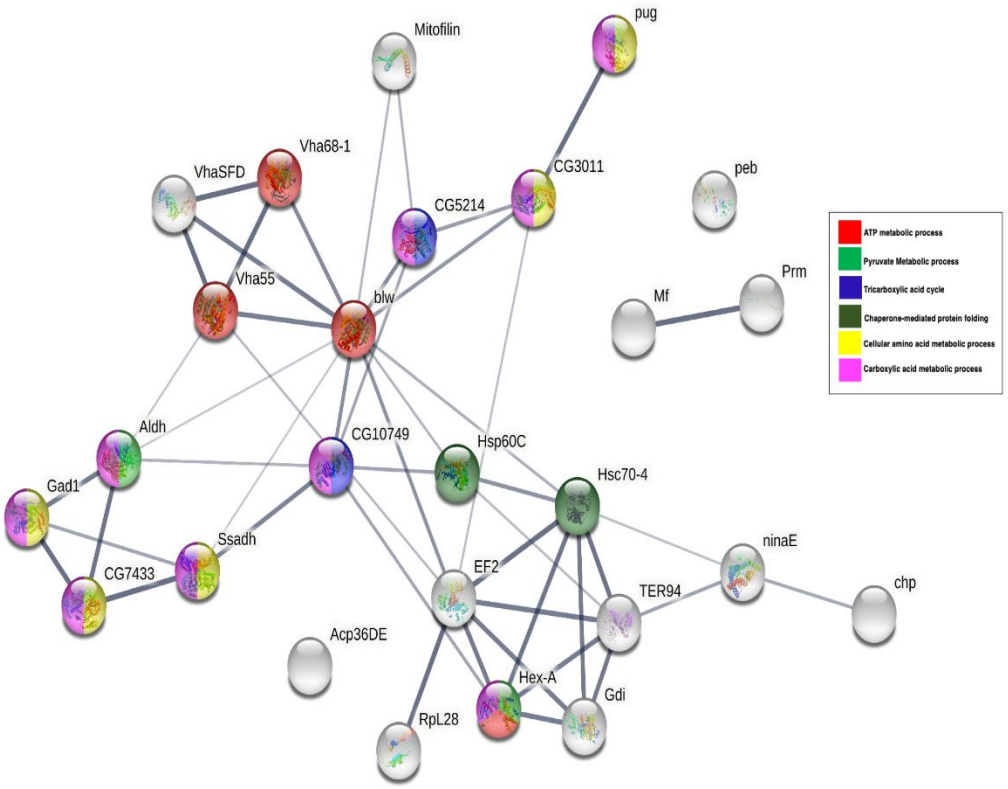

Figure 8 – Protein-protein interaction network of mitochondrial proteins that have decreased abundance in response to one week of exercise in D. melanogaster. Network analysis by the STRING v.11.0. software, with functional enrichment of P < 2.75e-08.

Gene Ontology Biological Process (GO:BP) enrichment analysis with the STRING database v.11.0. identified that among the proteins with significantly decreased expression in response to exercise there was enrichment for a diverse range of metabolic pathways (**Table 5**). These pathways include: the ATP metabolic process, the TCA cycle, the pyruvate metabolic process, the cellular amino acid metabolic process, the carboxylic acid metabolic process, and chaperone-mediated protein folding.

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Table 5 – Proteins identified from the mitochondrial fraction with decreased expression in response to exercise, with Gene Ontology Biological Process enrichment, as determined by STRING database v.11.0. analysis. Enrichment for a diverse range of metabolic processes is identified among the proteins with decreased expression. RAR, relative abundance ratio; FC, fold change.

Description	Accession	Significance (-log10P)	RAR	Log2 FC
ATP metabolic process				
ATP synthase subunit alpha mitochondrial	P35381	84.91	0.62	-0.68
TCA cycle				
GM01350p	Q9VGQ1	32.66	0.58	-0.78
Malate dehydrogenase	Q9VKX2	56.18	0.62	-0.68
Pyruvate metabolic process				
Aldehyde dehydrogenase	Q9VLC5	38.53	0.59	-0.76
Cellular amino acid metabolic process				
Succinate-semialdehyde dehydrogenase	Q9VBP6	77.81	0.63	-0.67
Gamma-aminobutyric acid transaminase isoform A	Q9VW68	200	0.52	-0.94
Serine hydroxymethyltransferase	Q9W457	200	0.66	-0.59
Carboxylic acid metabolic process				
GM01350p	Q9VGQ1	32.66	0.58	-0.78
Aldehyde dehydrogenase	Q9VLC5	38.53	0.59	-0.76
Malate dehydrogenase	Q9VKX2	56.18	0.62	-0.68
Serine hydroxymethyltransferase	Q9W457	200	0.66	-0.59
Succinate-semialdehyde dehydrogenase	Q9VBP6	77.81	0.63	-0.67
Gamma-aminobutyric acid transaminase isoform A	Q9VW68	200	0.52	-0.94
Chaperone-mediated protein folding				
Heat shock protein cognate 4 isoform G	C7LA75	153.53	0.58	-0.8
60 kDa heat shock protein homolog 2 mitochondrial	Q9VMN5	200	0.55	-0.87
Other mitochondrial proteins				
MICOS complex subunit MIC60	A0A0B4KGN2	27.65	0.64	-0.65

Discussion:

Lifetime exercise has worse outcomes than targeted exercise interventions in male and female Drosophila.

A consistent theme in our findings was the poor lifespan outcomes for both male and female *Drosophila* that were exercised throughout their lifetime, as opposed to targeted interventions (**Figure 1A-C, F and Figure 2A-D**), or in some instances no exercise at all (**Figure 1D and Figure 2E**). It could be suggested that lifetime exercise of the flies could produce injury or exhaustion leading to early mortality. In the case of single female flies, where no exercise at all had better lifespan outcomes than lifetime exercise, this theory is worth consideration.

Mechanistically, it is well-understood that acute exercise induces oxidative stress through generation of reactive oxygen species (ROS), which through the mitohormetic explanation induce healthy levels of activity in cellular antioxidant responses[37]. The field has now moved away from the idea of oxidative stress being only detrimental with regards to ageing with a more nuanced argument around low levels of oxidative stress and ageing [38]. However, it is also recognised that excessive levels of oxidative stress can contribute to the ageing process, so it may be the case that over-exercise, lifetime exercise in this instance, results in detrimental levels of oxidative stress being produced as a cellular response, ultimately

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contributing to the early mortality of these flies[39,40]. As with every drug, *the dose makes the poison*.

It is noteworthy that, at 30-days in the lifespan assay, there is a large difference in the probability of survival for grouped male and female flies that were exercised throughout their lifetime, compared with those that were subjected to targeted exercise intervention (**Figure 1A-C** and **Figure 2A-C**). This suggests that excessive, lifetime exercise, can increase the risk of early life mortality in the flies. Then, from the 30-day mark onward the probability of survival in these groups falls to similar levels, which indicates that much of the detrimental effects of consistent exercise are taking place early in the lifespan of the flies.

Late life exercise produces a rapid improvement in climbing assay scores compared with lifetime exercise for grouped male Drosophila

Middle life exercise, late life exercise and no exercise all produced significantly different climbing assay scores compared with lifetime exercise for grouped male *Drosophila* (**Figure 3**). When the difference seen between the 'late life vs lifetime' and 'no exercise vs lifetime' graphs is considered, it is striking given that the 'late life exercise group' had only been subject to three days of exercise to this point. One possible explanation for this large swing in climbing scores in response to just three days of exercise is that there is a rapid, adaptive response to exercise. It has previously been observed that male Drosophila have a greater adaptive response to exercise than females, in age-matched 5-day-old flies[41]. It could be the case that the male flies exercised later in life are also exhibiting a rapid adaptative response to a similar, short amount of exercise training that is no longer obvious after longer-term exercise.

Succinate-linked respiration is elevated in exercised flies

We show that daily exercise in *D. melanogaster* significantly increases oxygen flux when succinate is supplied, if compared with non-exercised flies (**Figure 4**). Succinate, a complex II substrate, is less efficient at producing ATP than complex I associated substrates (pyruvate and malate) as complex II does not pump protons that contribute to the electrochemical gradient. However, when ATP demand is high, such as during exercise, succinate may be an important substrate to help increase the ETC efficiency due to complex I substrates being more rapidly diminished.

Succinate has been previously shown to be a respiratory substrate utilised during stress[42,43]. In the bumble bee, *Bombus terrestris*, succinate oxidation has been shown to increase in flight muscle mitochondria by two-fold after a one-hour flight[42]. It is well established that exercise can induce an acute stress response; this resonates with our findings and the association between exercise, increased succinate oxidation and complex II activity.

Reduced spare respiratory capacity in exercised Drosophila may promote longevity.

Exercised flies had a significantly lower maximum electron transport chain capacity (ET capacity) compared to non-exercised flies (p-value = 0.002). Furthermore, the spare respiratory capacity (SRC), described as the mitochondrial capacity to produce ATP beyond routine respiration, was higher in the non-exercised flies[44]. This suggests that non-exercised flies are using a lower percentage of their maximum respiratory capacity to maintain routine

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respiration. This is in contrary to previous studies as exercise is acknowledged to improve mitochondrial function[45].

One explanation for the lower SRC in the exercised flies could be due to acute stress induced by exercise, as low SRC has been associated with poor adaptation to stress and an inability to meet ATP demands[44]. However, the ability to meet the energetic requirements of the cell by utilising oxidative phosphorylation to the fullest extent before resorting to anaerobic means could be considered advantageous later in life in that 'if you don't use it you lose it'. Measurements are needed of acute and longer term exercise cohorts, for comparison.

Proteins from the electron transport chain are significantly upregulated in response to exercise.

Proteomic analysis of mitochondria from flies that had been exercised for one week, 1 – 4 days post-eclosion showed higher quantities of mitochondrial electron transport chain proteins (**Figure 6** and **Table 4**). This could be a means of maximising the efficiency of the aerobic respiration that initially takes place during exercise during transition to anaerobic respiration[47]. Enzymatic activities of the electron transport chain are decreased during the ageing process as markers of oxidative stress increase[48]. This may be a simplistic view since Tavallaie *et al.* reported that administration of a moderate inhibitor of Complex IV promoted mitochondrial fitness in C57BL/6J mice, suggesting this may be used to mitigate against metabolic syndrome of ageing in humans[49]. It is possible that an upregulated electron transport chain corresponds to greater mitochondrial fitness, which would be beneficial in the context of ageing.

Multiple metabolic pathways are downregulated in response to exercise.

Enrichment analysis of proteins that were downregulated in the mitochondrial fraction after exercise pointed to a broad range of metabolic pathways (**Figure 8** and **Table 5**). While it is difficult to suggest a direct link between these pathways and their decreased activity in exercise, the variety of pathways identified could simply reflect the cellular conservation and resource redirection that takes place during exercise induced stress. The enrichment of chaperone mediated protein folding proteins, specifically heat shock protein cognate 4 isoform G and 60 kDa heat shock protein homolog 2 mitochondrial, may reflect a reduced rate of protein synthesis which would also support this. The identification of heat shock proteins is interesting as these are connected with failed proteostasis, one of the hallmarks of ageing[12,50].

Conclusion:

We find that targeted exercise as opposed to lifetime exercise, produces better survival outcomes in male and female *Drosophila*. Exercise has a rapid and significant effect on mitochondrial physiology seen through changes in the ETC of fruit flies. Through proteomic analysis we have found that components of the electron transport chain are upregulated in response to exercise, while a variety of other metabolic pathways show decreased expression. We suggest that exercise causes increased utilisation of mitochondrial pathways thus leading to better healthspan.

Author contributions

BE and YW performed data analysis, performed the experiments, and wrote the manuscript, TLI performed experimental work and helped prepare the manuscript, GK performed STRING analyses and helped prepare the manuscript, AB did the initial proteomics analyses, CS generated respirometry data and helped with writing, NM generated the flies used for these experiments and was consulted on all aspects of the fly work, LC directed the research, supervised experiments, provided reagents and prepared the manuscript.

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