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1 **Resistance training rejuvenates the mitochondrial methylome in aged human skeletal**
2 **muscle**

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37 NONSTANDARD ABBREVIATION LIST

38	CpG:	5'-Cytosine-phosphate-Guanine-3'
39	DMCs:	Differentially methylated CpGs
40	DNMT:	DNA methyltransferase
41	HSP:	mitochondrial heavy strand promoter
42	LSP:	mitochondrial light strand promoter
43	mCSA:	muscle cross-sectional area
44	mDNA:	mitochondrial DNA
45	MT-CO1:	mitochondrially-encoded Cytochrome C Oxidase I
46	MT-CYB:	mitochondrially-encoded Cytochrome B
47	MT-ND5:	mitochondrially-encoded NADH:Ubiquinone Oxidoreductase Core Subunit 5
48	MT-ND6:	mitochondrially-encoded NADH:Ubiquinone Oxidoreductase Core Subunit 6
49	MT-RNR2:	mitochondrially-encoded 16S rRNA
50	RRBS:	Reduced Representation Bisulfite Sequencing
51	rRNA:	ribosomal RNA
52	RT:	Resistance training
53	TET:	ten-eleven translocation enzymes
54	TFAM:	Transcription Factor A, Mitochondrial
55	VL:	vastus lateralis

56 ABSTRACT
57 Resistance training (RT) dynamically alters the skeletal muscle nuclear DNA methylome.
58 However, no study has examined if RT affects the mitochondrial DNA (mtDNA) methylome.
59 Herein, ten older, Caucasian untrained males (65±7 y.o.) performed six weeks of full-body RT
60 (twice weekly). Body composition and knee extensor torque were assessed prior to and 72 hours
61 following the last RT session. Vastus lateralis (VL) biopsies were also obtained. VL DNA was
62 subjected to reduced representation bisulfite sequencing providing excellent coverage across the
63 ~16-kilobase mtDNA methylome (254 CpG sites). Biochemical assays were also performed, and
64 older male data were compared to younger trained males (22±2 y.o., n=7, n=6 Caucasian & n=1
65 African American). RT increased whole-body lean tissue mass (p=0.017), VL thickness (p=0.012),
66 and knee extensor torque (p=0.029) in older males. RT also affected the mtDNA methylome, as
67 63% (159/254) of the CpG sites demonstrated reduced methylation (p<0.05). Several mtDNA sites
68 presented a more "youthful" signature in older males after RT in comparison to younger males.
69 The 1.12 kilobase mtDNA D-loop/control region, which regulates replication and transcription,
70 possessed enriched hypomethylation in older males following RT. Enhanced expression of
71 mitochondrial H- and L-strand genes and complex III/IV protein levels were also observed
72 (p<0.05). While limited to a shorter-term intervention, this is the first evidence showing RT alters
73 the mtDNA methylome in skeletal muscle. Observed methylome alterations may enhance
74 mitochondrial transcription, and RT evokes mitochondrial methylome profiles to mimic younger
75 men. The significance of these findings relative to broader RT-induced epigenetic changes need
76 to be elucidated.

77 Keywords: mitochondrial DNA, methylation, resistance training, aging

78 INTRODUCTION

79 Resistance training increases strength and muscle mass, and these adaptations have been
80 attributed to various mechanisms (e.g., an increase in satellite cell number, ribosome density, etc.).
81 Critically, molecular adaptations acutely induced by exercise precede well-documented
82 adaptations that occur with chronic training. In this regard, several reports have noted that single
83 exercise bouts transiently orchestrate the up- and down-regulation of hundreds of mRNA
84 transcripts in skeletal muscle (reviewed in (1)). These transcriptional events are complex and
85 involve the coordinated actions of histone-modifying enzymes, transcription factors,
86 transcriptional co-activators, and one of three RNA polymerase enzymes.

87 DNA methylation is a critical mechanism that regulates mRNA transcription (2). This
88 process involves a methyl group being transferred to the C-5 position of the cytosine ring, with
89 >98% of methylation occurring at cytosine guanine dinucleotide pairing sites (i.e., CpG sites).
90 DNA methylation is facilitated by DNA methyltransferase (DNMT) enzymes (3), and increased
91 methylation levels in a promoter or enhancer region negatively affect mRNA transcription by
92 either: i) impairing transcription factor binding, and/or ii) compacting DNA and making it
93 transcriptionally inaccessible. Recent enthusiasm has surrounded how exercise alters the collective
94 DNA methylome in skeletal muscle (4). Barres et al. (5) provided the first evidence, at the
95 candidate gene level, to suggest alterations in DNA methylation across canonical metabolic genes
96 in skeletal muscle can occur within hours of a single high-intensity aerobic exercise session.
97 Moreover, the changes in methylation inversely correlated with mRNA expression in the
98 corresponding genes. Subsequently, novel genome-wide methylation (methylome) studies in
99 human skeletal muscle have demonstrated that resistance exercise training (6, 7) and acute high
100 intensity running exercise (8) elicit DNA hypomethylation and the upregulation of genes related
101 to actin/cytoskeletal, extracellular matrix, growth-related pathways, and/or metabolic pathways.
102 These same studies have also shown that, following an earlier period of resistance training and
103 detraining, skeletal muscle DNA demonstrates hypomethylation. Importantly, some genes retain a
104 hypomethylated signature following training-induced hypertrophy, even during a period of
105 detraining as muscle mass returned to pre-training levels. Moreover, these genes were ‘enhanced’
106 during retraining as a consequence of earlier training, suggesting human skeletal muscle possesses
107 an epigenetic memory of earlier exercise (or ‘epi-memory’) (9). The biological process of aging
108 seems to have the opposite effect on the skeletal muscle DNA methylome whereby
109 hypermethylation seemingly accumulates (10-13). However, increased physical activity (11) and
110 resistance exercise (14) have been shown to reverse hypermethylated profiles with age to more
111 hypomethylated signatures.

112 Although resistance training clearly affects molecular mechanisms related to skeletal
113 muscle hypertrophy, the effects of resistance training on mitochondrial adaptations are less clear.
114 Studies in younger adult populations have reported that markers indicative of mitochondrial
115 volume increase, decrease, or do not change in response to several weeks of resistance training
116 (15, 16). We recently reported that 10 weeks of resistance training doubles skeletal muscle citrate
117 synthase activity (a surrogate of mitochondrial volume) in older adults (17). Others have also
118 reported that markers reflective of improved mitochondrial function occur in older adults after 14
119 weeks of resistance training (18). Thus, it is plausible that increases in mitochondrial biogenesis
120 and improvements in mitochondrial function may occur in an age-dependent fashion where robust
121 effects are more evident in older versus younger adults. Just as with the nuclear genome, the
122 mitochondrial genome can undergo dynamic DNA methylation and demethylation (19). Earlier
123 research in this area suggested alterations in mitochondrial DNA (mtDNA) methylation was

124 relatively low in comparison to the dynamic changes that occur with nuclear DNA methylation
125 (20). Nonetheless, other studies have since suggested that the mtDNA methylome can be
126 transiently modulated through various perturbations. For instance, Wong et al. (21) used DNA
127 pyrosequencing to demonstrate that mtDNA methylation patterns and mitochondrial DNMT3a
128 levels are abnormal in the skeletal muscle and spinal cord of transgenic mice that develop
129 amyotrophic lateral sclerosis. Moreover, Patil et al. (22) recently demonstrated that mtDNA
130 methylation patterns differed in cancerous versus non-cancerous human cell lines. However, given
131 the infancy of this research, it is unclear as to how changes in mtDNA methylation affect
132 mitochondrial physiology.
133 In spite of the discoveries mentioned above in relation to the nuclear methylome and
134 exercise training, no studies to date have examined how exercise training affects mtDNA
135 methylation patterns in skeletal muscle. This lack of data is, in part, due to methylome studies
136 undertaking array profiling of CpG methylation. Alternatively stated, few human exercise studies
137 have undertaken bisulfite sequencing of skeletal muscle that allows in-depth analysis of mtDNA
138 methylation patterns. Therefore, the current study contained multiple objectives. First, we used a
139 genome-wide DNA bisulfite sequencing strategy (Reduced Representation Bisulfite Sequencing,
140 or RRBS) to determine how six weeks of resistance training altered mtDNA methylation patterns
141 in skeletal muscle of older, previously untrained males. Notably, younger resistance-trained males
142 were also included in this analysis as a comparator group. Next, we determined if the alterations
143 observed at the mtDNA methylome level were associated with corresponding changes in
144 mitochondrial gene expression as well as mitochondrial protein complexes and citrate synthase
145 activity (a marker of mitochondrial volume). Finally, we sought to determine if resistance training
146 was able to rejuvenate the mtDNA methylome profiles of older males to profiles observed in
147 younger males.
148
149
150 MATERIALS AND METHODS
151 *Ethical approval*
152 This study was a secondary analysis of two studies approved by the Institutional Review
153 Board at Auburn University. The first protocol (Protocol # 19-249 MR 1907) involved examining
154 the effects of resistance training with daily peanut protein supplementation or no supplementation
155 on skeletal muscle hypertrophy in untrained, older adults between the ages of 50 to 75 years
156 (NCT04015479). Ten older males from the 6-week cohort (n=5 per group) were examined herein.
157 Two-way repeated measures ANOVAs indicated that none of the body composition or assayed
158 biomarkers were affected by peanut protein supplementation (interaction p-values: lean body
159 mass, p=0.952; vastus lateralis (VL) thickness, p=0.543; knee extensor peak torque, p=0.893; all
160 qPCR and Western blot markers, p>0.200; CS activity, p=0.335). The second study (younger
161 participants) involved examining the effects of unilateral resistance training on muscle
162 hypertrophy outcomes in seven previously trained young adult males (Protocol # 19-245 MR
163 1907). Training status for this study was determined by two criteria: i) self-reported resistance
164 training >1 year at least 3 times weekly and; ii) a tested barbell back squat of ≥1.5× bodyweight
165 (estimated from a 3 repetition maximum [3RM] test) in accordance to standards designated by the
166 National Strength and Conditioning Association. At the conclusion of the screening visit,
167 participants were asked to maintain their current nutritional practices and to cease all training
168 outside of the study. Resting baseline biopsies from these participants (obtained 72 hours
169 following their last exercise bout, and prior to the initiation of the study) were used as a comparator

group to the older participants to determine if resistance training rejuvenated the mtDNA methylome.

Inclusion criteria for both studies required participants to abstain from nutritional supplementation (e.g., creatine monohydrate, protein supplements) one month prior to testing. Participants from both studies had to be free of overt cardio-metabolic diseases (e.g., type II diabetes, severe hypertension, heart failure) or conditions that precluded the collection of a skeletal muscle biopsy. All participants provided verbal and written consent to participate in each respective study, and both studies conformed to standards set by the latest revision of the Declaration of Helsinki. Data herein included 10 older male participants (age = 65 ± 7 years old; mean ± SD), and 7 previously trained younger adult males (22 ± 2 years old, self-reported resistance training experience of 5 ± 1 years).

Resistance training program for older participants

The training program for older males has been previously described (23). Briefly, participants underwent supervised resistance training twice weekly, on non-consecutive days, for six weeks. Each session consisted of five exercises including leg press, leg extensions, lying leg curls, barbell bench press, and cable pull downs. For each exercise, participants performed three sets of 8-12 repetitions to volitional fatigue with at least one minute of rest in between sets. At the end of each set, participants were asked to rate the level of difficulty (0 = easy, 10 = hard). If values were below 7, weight was added to increase effort for the next working set. If values were 10, or the participant could not complete the set, weight was removed prior to the next working set. Participants were encouraged to be as truthful as possible when assessing difficulty. The intent of this training method was to challenge participants where perceived exertion after each set was between a 7-9 rating. This method allowed us to ensure that training effort was maximized within each training session, and that the participants were successfully implementing progressive overload in an individualized fashion.

Testing sessions

For younger and older participants, the testing sessions described below occurred during morning hours (05:00–09:00) following an overnight fast. For older males, Pre-testing occurred ~2-5 days prior to the first day of resistance training, and Post-testing occurred 72 hours following the last training bout. The younger males performed all of the same tests described above between 05:00-11:00.

Prior to testing batteries, participants submitted a urine sample (~5 mL) to assess urine specific gravity (USG) using a handheld refractometer (ATAGO; Bellevue, WA, USA). USG in all participants were <1.020 indicating sufficient hydration (24). Height and body mass were assessed using a digital column scale (Seca 769; Hanover, MD, USA), and values were recorded to the nearest 0.1 kg and 0.5 cm, respectively. Participants then had their bone-free lean/soft tissue mass (LSTM) and fat mass determined by a full-body dual-energy x-ray absorptiometry (DXA) scan (Lunar Prodigy; GE Corporation, Fairfield, CT, USA). The same investigator completed all DXA scans. According to previous data published by our laboratory (25), the same-day test-calibrate-retest reliability on 10 participants produced an intra-class correlation coefficient (ICC) of 0.998 for LSTM. After DXA scans, a cross-sectional image of the right thigh at 50% of the femur length was acquired using a peripheral quantitative computed tomography (pQCT) scanner (Stratec XCT 3000, Stratec Medical, Pforzheim, Germany). Scans were acquired using a single 2.4 mm slice thickness, a voxel size of 0.4 mm and scanning speed of 20 mm/sec. Images were

216 analyzed for total muscle cross-sectional area (mCSA, cm^2) using the pQCT BoneJ plugin freely
217 available through ImageJ analysis software (NIH, Bethesda, MD, USA). All scans were performed
218 and analyzed by the same investigator, and the ICC was previously determined for mCSA to be
219 0.990 (*unpublished data*). Following pQCT assessments, right leg vastus lateralis ultrasound
220 assessments were performed using a 3-12 MHz multi-frequency linear phase array transducer
221 (Logiq S7 R2 Expert; General Electric, Fairfield, CT, USA) to determine muscle thickness.
222 Participants were instructed to stand and displace bodyweight more to the left leg to ensure the
223 right leg was relaxed. Measurements were standardized by placing the transducer at the midway
224 point between the inguinal crease and proximal patella. The same technician performed all
225 ultrasounds. According to previous data from our laboratory, the 24-hour test-retest reliability for
226 muscle thickness assessment on 11 participants resulted in an ICC of 0.983.
227 Right leg vastus lateralis muscle biopsies were then obtained with a 5-gauge needle as
228 previously described (26). Following biopsies, tissue was rapidly teased of blood and connective
229 tissue, wrapped in pre-labeled foils, flash frozen in liquid nitrogen, and subsequently stored at -
230 80°C for further molecular analyses.
231 In older males, right leg knee extensor peak torque testing occurred ~1-3 days prior to the
232 muscle biopsy at the pre time point, whereas this test occurred approximately 30 minutes prior to
233 the biopsy at the post-test time point. In younger males, this test occurred approximately 10
234 minutes following the biopsy. During testing, participants were fastened to an isokinetic
235 dynamometer (Biodex System 4; Biodex Medical Systems, Inc., Shirley, NY, USA). Each
236 participant's knee was aligned with the axis of the dynamometer, and seat height was adjusted to
237 ensure the hip angle was approximately 90°. Prior to peak torque assessment, each participant
238 performed a warmup consisting of submaximal to maximal isokinetic knee extensions. Participants
239 then completed five maximal voluntary isokinetic knee extension actions at 60°/s. Participants
240 were provided verbal encouragement during each contraction. The isokinetic extension resulting
241 in the greatest value for peak torque was used for analyses.
242
243 *Molecular analyses of skeletal muscle.*
244 *DNA isolation.* Muscle samples stored in foils were removed from -80ºC and placed on a
245 liquid nitrogen-cooled ceramic mortar. Tissue was crushed using a ceramic pestle, and ~10 mg
246 was obtained for DNA isolation using the commercially available DNeasy Blood & Tissue Kit
247 (Qiagen; Venlo, The Netherlands; catalog #: 69504) as per the manufacturer's recommendations.
248 DNA pellets were reconstituted in a buffer provided by the kit, and concentrations were determined
249 in duplicate at an absorbance of 260/280 nm (1.81 ± 0.08) using a desktop spectrophotometer
250 (NanoDrop Lite; Thermo Fisher Scientific; Waltham, MA, USA). DNA was then shipped to a
251 commercial vendor (EpiGentek Group Inc.; Farmingdale, NY, USA) for RRBS as described
252 below.
253 *DNA bisulfite conversion and RRBS.* Samples were received by the commercial vendor on
254 dry ice, and were subjected to enzymatic digestion (MSP1 + TaqI); specifically, 300 ng of DNA
255 from each participant was digested for 2 hours with MSP1 enzyme (20U/sample) at 37°C followed
256 by 2 hours with TaqαI (20U/sample) at 65°C. Digested DNA <300 base pair fragments were
257 collected for bisulfite treatment, and bisulfite conversion was performed with the Methylamp DNA
258 Bisulfite Conversion Kit (Epigentek; catalog #: P-1001). The efficiency of bisulfite-treated DNA
259 was determined by real-time PCR using two pairs of primers where the first pair targeted bisulfite-
260 converted beta-actin (BACT), and the second pair targeted unconverted Glyceraldehyde-3-
261 Phosphate Dehydrogenase (GAPDH) for the same bisulfite-treated DNA samples. Library

preparation then ensued, and Bioanalyzer QC and KAPA library quantification were performed thereafter. Sample libraries (20 nM) were subjected to multiplex next generation sequencing using an Illumina HiSeq4000 (Illumina Inc.; San Diego, CA, USA). Quality control on raw reads was performed using FASTQC, version 0.11.8 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/), and an HTML report was generated for each data set. Quality and adapter trimming was performed on the raw reads using Trim Galore, version 0.5.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trim Galore performs the following trimming steps: i) low-quality read removal (Sanger Phred score of 20 or lower), ii) trimming of the 3' Illumina adapter (any signs of AGATCGGAAGAGC), and iii) removal of trimmed reads shorter than 20 bp. Trimmed reads were mapped to the UCSC homo sapiens (human) genome sequence (version GRCh38) using a methylation-aware mapper, bismark, version 0.203.0 (27). Bismark utilizes Bowtie, version 2.2.5 (28), with the option "--directional" for targeted bisulfite sequencing libraries and option "--pbat" for post-bisulfite prepared RRBS libraries. For each sample, a summary HTML report was generated, which included alignment and cytosine methylation statistics. Samtools, version 0.1.9 (29), was utilized to sort the SAM file produced by bismark and remove the duplicate reads due to PCR amplification. Methylation information was extracted from the final bismark mapping result at the base resolution where a minimal read coverage score of 10 and minimal quality score of 20 at each base position are applied. The resulting CpG sites were filtered based on coverage and merged for comparative analysis using MethylKit package (https://github.com/al2na/methylKit) in R (version 4.0.3). Only CpG sites that met both read coverage and read base quality score thresholds in all samples, across all conditions were utilized for downstream analyses. This meant that for all comparisons utilized in older adult trained versus untrained data sets, 254 CpG sites were covered within the mtDNA. For inclusion of the younger trained adult data set, this number reduced to 253 CpG sites in the mtDNA as a consequence of our quality score and read coverage thresholds. Principle Component Analysis plots were subsequently performed to determine group-level quality control. Differentially Methylated Regions (DMRs) and Differentially Methylated CpGs (DMCs) were processed in a similar manner. However, for DMR analysis, data sets were first chunked into 100bp windows with a step size of 100bp. Differential analysis was then performed using MethylKits *calculateDiffmeth* function and logistic regression to calculate differential P values which were then transformed to Q values using the SLIM method (30), and DMRs/DMCs were extracted. We explored the mtDNA data set by setting differential methylation changes of 3 and 5% with an adjusted P-value of less than 0.05 (FDR < 0.05), as recent work has suggested that only small differences are likely to be observed in the mtDNA genome (31), given it is a lowly methylated region in basal/homeostatic samples (32).

RNA isolation with Trizol and targeted qPCR. Approximately 10 mg of muscle was placed in 500 µl of Ribozol (Ameresco; Solon, OH, USA), and RNA isolation proceeded following the manufacturer's instructions. RNA concentrations were determined in duplicate using a NanoDrop Lite (Thermo Fisher Scientific), and cDNA (2 µg) was synthesized using a commercial qScript cDNA SuperMix (Quanta Biosciences; Gaithersburg, MD, USA). Real-time qPCR was performed in a thermal cycler (Bio-Rad Laboratories; Hercules, CA, USA) using SYBR green-based methods and gene-specific primers were designed with publically-available software (Primer3Plus; Cambridge, MA, USA). For all primer sets, pilot qPCR reactions and melt curves indicated that only one amplicon was present. The forward and reverse primer sequences of all genes are listed in Table 2. Fold change values were performed using the $2^{\Delta\Delta\text{-}Cq}$ method where $2^{\Delta\text{-}Cq}$ = 2^(housekeeping gene (HKG) Cq - gene of interest Cq), and $2^{\Delta\Delta\text{-}Cq}$ (or fold change) = ($2^{\Delta Cq}$

value/$2^{\Delta Cq}$ average of Pre values). GAPDH was used as the reference/HKG gene, and GAPDH Cq values were stable with training in the older participants (Pre: 26.25 ± 1.13, mean coefficient of variation = 0.23%, Post: 26.48 ± 0.50, mean coefficient of variation = 0.23%; p=0.523 between Pre and Post).

INSERT TABLE 2 HERE

Western blotting (mitochondrial complexes and TFAM protein expression). Muscle stored in foils were removed from -80ºC and placed on a liquid nitrogen-cooled ceramic mortar. Tissue was crushed using a ceramic pestle, and ~20 mg was placed in 1.7 mL microcentrifuge tubes prefilled with general cell lysis buffer (25 mM Tris, pH 7.2, 0.5% Triton X-100, 1x protease inhibitors). Samples were homogenized on ice using hard-plastic pestles, and centrifuged at 1,500 g for 10 minutes at 4°C. Supernatants were collected and placed in new 1.7 mL microtubes on ice. Supernatant protein concentrations were determined using a commercially available BCA kit (Thermo Fisher Scientific; Waltham, MA, USA) according to manufacturer's instructions. Afterwards, supernatants were prepared for Western blotting using 4x Laemmli buffer and distilled water (diH_2O) at a concentration of 1 µg/µL, and denatured for 5 minutes at 100°C prior to being frozen at -80°C until Western blotting. On the day of Western blotting, prepared samples (15 µL) were pipetted onto gradient SDS-polyacrylamide gels (4%–15% Criterion TGX Stain-free gels; Bio-Rad Laboratories), and electrophoresis commenced at 180 V for 50 minutes. Following electrophoresis, proteins were transferred to pre-activated PVDF membranes (Bio-Rad Laboratories) for two hours at 200 mA. Gels were then Ponceau stained for five minutes, washed with diH_2O for one minute, dried for one hour, and digitally imaged with a gel documentation system (ChemiDoc Touch; Bio-Rad Laboratories). Following Ponceau imaging, membranes were re-activated in methanol, blocked with nonfat milk for one hour (5% w/v diluted in Tri-buffered saline with 0.1% Tween 20, or TBST), washed three times in TBST only (5 minutes per wash). Membranes were then incubated for 24 hours with the following antibodies (1:1000 v/v dilution in TBST): i) mouse anti-human OxPhos cocktail (Abcam; Cambridge, MA, USA; catalog#: ab110411), ii) rabbit anti-human TFAM (Abnova; Taipei, Taiwan; catalog #: H00007019-D01P), and iii) COX IV (Cell Signaling Technology; Danvers, MA, USA; Cat# 4850). Notably, COX IV was assayed independently given that the OxPhos antibody cocktail is incapable of assaying this marker in samples that are boiled prior to Western blotting; see Mesquita et al. (33) where we have performed Western blotting on human muscle tissue in a similar manner.

Following primary antibody incubations, membranes were washed three times in TBST only (5 minutes per wash), and incubated for one hour with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Cell Signaling Technology; catalog #'s: 7076 and 7074). Membranes were then washed three times in TBST only (five minutes per wash), developed using chemiluminescent substrate (EMD Millipore; Burlington, MA, USA), and digitally imaged using a gel documentation system (ChemiDoc Touch; Bio-Rad Laboratories). For all Western blot targets, raw target band densities were obtained using associated software (Image Lab v6.0.1; Bio-Rad Laboratories), and these values were divided by Ponceau densities at 25-100 kD. Target/Ponceau density ratios were then divided by the grand mean of older participants at the Pre time point in order to obtain relative protein expression values. For Western blotting, 9 of 10 older participants and 6 of 7 younger males were assayed due to tissue limitations.

Determination of muscle citrate synthase activity. Muscle citrate synthase activity levels were determined in duplicate on supernatants obtained from muscle described in the Western

354 blotting section; notably, these methods are similar to previous methods used by our laboratory
355 (34, 35). The assay principle is based on the reduction of 5,50-dithiobis(2- nitrobenzoic acid)
356 (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm) coupled to the reduction of acetyl-
357 CoA by the citrate synthase reaction in the presence of oxaloacetate. Briefly, 12.5 µg of skeletal
358 muscle protein obtained from supernatants was added to a mixture composed of 0.125 mol/L Tris–
359 HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L DTNB. All duplicate reactions occurred
360 in 96-well plates, reactions were initiated by the addition of 5 µL of 50 mmol/L oxaloacetate per
361 well, and the absorbance change was recorded for 60 seconds in a spectrophotometer (Synergy
362 H1; BioTek; Winooski, VT, USA). Again, 6 of 7 younger males were assayed due to tissue
363 limitations.
364
365 *Statistics*
366 Phenotype and select molecular data were compared from pre to post training in older
367 males using dependent samples t-tests. Additionally, comparisons of data from older males at these
368 time points were made to younger males using independent samples t-tests. Methylation data were
369 analyzed from pre- to post-training in older males and between older and younger participants
370 using a variety of statistical methods that are described in greater detail in the results section.
371 Select dependent variables were also correlated using Pearson's correlation coefficients. All data
372 herein are presented in figures and tables as means ± standard deviation values unless stated
373 otherwise, and statistical significance was set at $p<0.05$.
374
375
376 RESULTS
377 *Training adaptations*
378 General training adaptations in older males and a comparison to younger males are
379 presented in Table 1. At the end of the six weeks of training, lean/soft tissue mass, vastus lateralis
380 thickness, knee extensor peak torque and mCSA increased in older males ($p=0.017$, $p=0.012$,
381 $p=0.029$, and $p=0.057$ respectively). However, post-training values in older males were still
382 significantly lower than values in younger, trained males ($p<0.05$ for all variables).
383
384 INSERT TABLE 1 HERE
385
386 *Mitochondrial DNA methylation*
387 The RRBS data set spanned 254 individual CpG sites mapping to the ~16 kb mtDNA
388 region of the human genome. Comparative analysis in older participants prior to and following
389 resistance training shows that 63% of these CpG sites (159/254) demonstrated a significant
390 reduction in methylation following training (FDR < 0.05; change ≥ 3%; Suppl. File 1A). Even at
391 a more stringent pre-to-post training change (≥ 5%), a large number of sites (~40%, 98/254 CpGs)
392 possessed a hypomethylated signature. Interestingly, with the same significance criteria, no CpG
393 sites increased in methylation following training. Human mtDNA methylation levels have
394 previously been suggested to be dependent on sequencing coverage biases (32). We clearly show
395 no differential read coverage issues between time points (Figure 1A), and read density had no
396 association with methylation levels in these conditions (Figure 1B).
397 We subsequently mapped methylation patterns across the 16 kb region and performed
398 differentially methylated region (DMR) analyses to identify loci that were prone to methylation
399 changes following resistance training. Using a 100 bp size sliding window model, with stringent

400 significance thresholds set (e.g. q value < 0.01, differential change of > 5% and with at least 3
401 contiguous 100 bp windows identified), we identified 4 DMRs (Suppl. File 1B). Interestingly, this
402 analysis identified a DMR spanning a larger 500 bp region whose origin mapped to that of the D-
403 loop/control region of the human mtDNA genome.
404
405 INSERT FIGURE 1 HERE
406
407 The entire control region spans a 1.12 kb locus containing a number of extra regulatory elements
408 including the hyper-variable region (HVR), a tertiary DNA fragment (7S DNA), control elements
409 (Mt5 and Mt3L) and TFAM binding sites (Figure 2A). Crucially, the 1.12 kb locus also contains
410 the light strand promotor (LSP), one of the two heavy strand promotors (HSP1), and the HSP2
411 promoter resides less than 100 bp away from this region (Figure 2A). Given that we identified a
412 DMR within this control region (Figure 1D), and this region regulates mtDNA replication and
413 transcription, we examined the locus that spans the mtDNA control region as well as the HSP2
414 region (from 16024 to 650, Figure 2A). In this region, we also identified differentially methylated
415 profiles of the CpGs following resistance training in older participants (Figure 2B). Four of five
416 CpG sites residing within close proximity to either HSP1, HSP2 or LSP showed a significant
417 reduction in methylation (FDR < 0.05, change of > 5%; Figure 2B). This suggests the control
418 region, and in particular the 5'-prime end, is largely hypomethylated following resistance training
419 in older participants.
420 Associating CpG site methylation data in older participants prior to and following training
421 against phenotype variables, we also identified several significant correlations (Figure 2C; Suppl.
422 File 2). CpG sites residing at positions 61 and 97 of the mtDNA genome showed significant inverse
423 correlations between CpG methylation and vastus lateralis thickness as well as whole-body fat
424 mass, respectively (Figure 2C). These same positions also showed association trends between
425 other phenotype variables within our data sets (albeit not significant; Suppl. File 2). Interestingly,
426 two sites (positions 106 and 16455) in our analyses displayed positive correlations between
427 methylation and phenotype variables. Methylation of site 106 strongly correlated with knee
428 extensor peak torque (p=0.01) and, to a lesser extent, muscle cross sectional area (p=0.03). We
429 also identified a trend for an inverse relationship between CpG site 16450 and knee extensor peak
430 torque (r=-0.39, p=0.08). Finally, methylation of position 16455 significantly correlated with both
431 whole-body fat free mass (p=0.02) and vastus lateralis thickness (p=0.03).
432 Next, we compared the methylation profiles of our older males prior to and following
433 training to younger males to ascertain whether training in the older participants restores mtDNA
434 methylation levels to youth like levels. A significant difference was observed between younger
435 and older males prior to training (p= 0.014; Figure 2D). However, methylation patterns of younger
436 participants showed no significant difference compared to older participants following training
437 (p>0.05; Figure 2D). Additionally, across the mtDNA genome, a highly comparable methylation
438 profile existed between younger and older males following training (Figure 2E). Collectively,
439 these data suggest resistance training restored the mtDNA methylome in older participants to
440 mimic a more youthful signature.
441
442 INSERT FIGURE 2 HERE
443
444 *Mitochondrial gene expression and protein complexes*

445 The majority of mtDNA genes are transcribed from the HSP2 region. The primary role of
446 HSP1 is to transcribe mitochondrial rRNA genes on the heavy strand. LSP1 transcribes ND6,
447 which is the only coding transcribing gene on the light strand. We therefore undertook qPCR to
448 assess mitochondrial gene expression of MT-CYB (cytochrome B), MT-ND5 (NADH
449 dehydrogenase 5) and CO1 (cytochrome c oxidase subunit I) as well as MT-RNR2
450 (mitochondrially-encoded 16S rRNA) and light strand ND6 (NADH dehydrogenase 6) mRNA
451 levels. Given TFAM binding sites were located close to the hypomethylated region identified in
452 the DMR analysis above we also measured TFAM gene expression. Resistance training in older
453 participants increased all assayed mitochondrial mRNA targets (MT-CYB, MT-ND6, MT-ND5,
454 and MT-CO1) as well as MT-RNR2 RNA levels ($p<0.05$) (Figure 3A), but not TFAM expression
455 (Figure 3B; $p > 0.05$). We also confirmed no change in TFAM protein levels ($p>0.05$; Figure 3C).
456 All of the genes analyzed demonstrated higher levels in the younger compared to older males at
457 both the pre- and post-training time points ($p<0.05$ for all targets). Furthermore, citrate synthase
458 activity assays were performed to assess mitochondrial volume (Figure 3D), and no change
459 occurred in older individuals with resistance training ($p=1.00$). Skeletal muscle protein levels of
460 electron transport chain complexes were analyzed, and resistance training in older participants
461 increased protein levels of complexes III and IV ($p<0.05$) (Figure 3E/F).
462
463 INSERT FIGURE 3 HERE
464
465 *Correlation of methylation with mRNA expression and protein levels*
466 We examined associations between CpG methylation patterns and alterations in gene
467 expression to explore the transcriptional consequence of the differentially methylated regions
468 identified. A clear inverse association existed between CpG site methylation (CpG sites residing
469 in our 1.12kb mtDNA loci) and mitochondrial gene expression (Figure 4A). Interestingly, our
470 analysis identified MT-ND6 and MT-RNR2 expression to be the two most commonly inversely
471 associated transcripts (Suppl. Fig 2A and 2B), with CpG site 162 showing the strongest inverse
472 association with MT-RNR2 expression ($r=0.59$, $p=0.005$) (Figure 4C). Across all analyses, CpG
473 site 16,329, which resides in HVR1, displayed the most consistent negative/inverse correlation
474 between methylation and gene expression (Suppl. File 3). Counterintuitively, but in keeping with
475 correlational analyses performed on our phenotype data sets, CpG site 16455 positively correlated
476 with expression of our analysed gene sets (Figure 4A/B, Suppl. Figure 2A, Suppl. File 3).
477 However, this CpG site is not positioned within any key regulatory locus.
478 We further correlated methylation with the abundance of complex III and IV proteins
479 (Figure 4A). Methylation of CpG sites 163 to 16329 (Figure A) demonstrated a strong inverse
480 association with complex III protein abundance ($P < 0.001$; Figure 4A; Suppl. File 4). CpG site
481 16129 demonstrated an inverse association ($r = -0.76$, $p=0.0002$; Figure 4E). In keeping with the
482 identification of the positive correlations between CpG methylation of site 106 and phenotypic
483 variables as well as gene expression, we identified a positive correlation ($r = 0.48$, $p=0.04$) between
484 the methylation of this site and complex IV protein abundance (Figures 4A/D), but such an
485 association was not evident with complex III protein abundance.
486
487 INSERT FIGURE 4 HERE
488
489 DISCUSSION

490 This study is the first to illustrate that resistance training leads to hypomethylation of the
491 mitochondrial genome in skeletal muscle. Moreover, resistance training seemingly restored
492 mtDNA methylation signatures in older males relative to younger, trained males. Pre- to post
493 training increases in mitochondrial mRNA and rRNA levels in older participants aligned with the
494 observation that the D loop/control region, which regulates mitochondrial transcription and
495 replication, demonstrated increased hypomethylation with training. There were also interesting
496 associations between mtDNA methylation patterns and various phenotypes. These findings are
497 discussed in greater detail below. From a healthy aging perspective, these data continue to suggest
498 resistance training has beneficial effects on certain aspects of mitochondrial physiology.
499 Skeletal muscle DNA methylation has been reported to increase with aging, and this
500 typically coincides with a decrease in the mRNAs of genes that exist downstream of methylated
501 regions (11, 14, 36-38). However, data are lacking with regard to how aging affects the
502 methylation status of the mitochondrial genome in skeletal muscle. D'Aquila et al. (39)
503 demonstrated that the methylation of the 12S rRNA region of the mitochondrial genome in PBMCs
504 increases with aging, and 9-year follow-up data illustrate that increased methylation in this region
505 is associated with increased mortality. The current data are in agreement with the findings of
506 D'Aquila and colleagues in that skeletal muscle from older participants prior to training displayed
507 increased mtDNA methylation levels compared to younger, trained participants. Moreover, these
508 methylation patterns coincided with lower mRNA and rRNA levels of various mitochondrial genes
509 as well as lower protein abundances of certain complexes in the older participants. Remarkably,
510 resistance training in older participants decreased mtDNA methylation patterns in certain regions.
511 Although longer-term exercise training has been shown to alter skeletal muscle DNA methylation
512 patterns in younger (7, 40) and middle-aged (41) participants, results from these studies suggest
513 that various genes can be hypo- or hypermethylated. Furthermore, none of these studies
514 interrogated the mtDNA methylation changes given that chip arrays lacking mtDNA probes were
515 utilized. The current findings agree in principle with a recent meta-analysis that examined 16
516 research studies and concluded that nuclear DNA methylation generally decreases with exercise
517 in older adults (42). Additionally, our data agree in principle with other findings that show
518 resistance exercise evokes nuclear genome hypomethylation in older human skeletal muscle (14).
519 However, our findings strongly extend the current literature given that it is the first to suggest
520 exercise training can lead to the hypomethylation of the mitochondrial genome, and DMR analysis
521 demonstrates this hypomethylation is particularly enriched in important regulatory regions for
522 mtDNA transcription and replication.
523 Prior to discussing the implications of the mtDNA methylation data, it is important to
524 appreciate how the nuclear and mitochondrial genomes differ. The nuclear genome contains
525 approximately 3 billion base pairs, encodes for just over 20,000 genes, and each gene typically
526 contains one segment of DNA separated by regions of non-coding DNA. mtDNA contains 16,569
527 base pairs, and encodes for 37 genes including 2 rRNAs, 22 tRNAs and 13 proteins. The
528 mitochondrial genome possesses a heavy strand (H-strand) and light strand (L-strand) where
529 polycistronic RNAs are transcribed from each strand, and subsequently cleaved and processed to
530 yield mitochondrial rRNAs, tRNAs and mRNAs (43, 44). Two transcription initiation sites exist
531 in a region termed the D-loop. These sites are termed heavy strand promoter 1 (HSP1) and light
532 strand promoter (LSP), and each is where H-strand and L-strand transcription initiation occurs,
533 respectively. Of the mtDNA regions that demonstrated enriched hypomethylation with resistance
534 training in older participants, the most interesting site identified was the D-loop/control region.
535 This finding suggests that resistance training promotes a favorable environment for increased

536 mitochondrial transcription and aligns with our findings of increased RNA levels H-strand genes
537 (MT-RNR2, MT-CO1, MT-CYB, MT-ND5) and an L-strand gene (MT-ND6) in older individuals
538 following training. While these findings are novel and provocative, it is unclear as to whether the
539 observed methylation and RNA adaptations in older individuals directly facilitated mitochondrial
540 adaptations. In this regard, citrate synthase activity levels, which are strongly associated with
541 mitochondrial volume, remained unaltered with training in older participants. Likewise, only select
542 mitochondrial proteins (specifically, complexes III and IV) were upregulated with training.
543 Although these findings seemingly suggest mitochondrial biogenesis did not occur with training,
544 it is critical to interpret these data in the context of assay logistics, and we have published a recent
545 review in this area (15). CS activity, as well as complex proteins, are normalized to total muscle
546 protein. Thus, in a muscle cell that grows and accretes protein during resistance training, no
547 change in these metrics from pre- to post-training indicate that mitochondrial expansion occurred,
548 but at a similar pace to cellular hypertrophy. In endurance training scenarios, there is less-to-
549 minimal myofiber hypertrophy in lieu of mitochondrial expansion. Thus, this ultimately results in
550 more robust increases in CS activity. Because of the hypertrophy-mitochondrial expansion
551 concurrence, data are mixed where some studies show a decrease in CS activity, some show no
552 change, and only two studies have shown an increase (15). It is notable, however, that our
553 laboratory has reported 10 weeks of resistance training robustly increased CS activity levels in
554 older participants (17). Moreover, others have reported that longer-term resistance training (~3
555 months) increases various aspects of mitochondrial function (e.g., respiration and/or complex
556 activities) in older participants (18, 45, 46). Mitochondrial adaptations involve a coordinated effort
557 between the nuclear and mitochondrial genomes given that most mitochondrial proteins are
558 encoded by the nuclear genome (15). Thus, the observed mtDNA methylation changes with
559 resistance training may precede certain longer-term mitochondrial adaptations.
560 We were also interested in determining whether TFAM mRNA or protein levels were
561 altered in older participants with resistance training given that TFAM is mitochondrial
562 transcription factor that binds to various regions in the D-loop and is critical for stimulating
563 transcription. There were no alterations in TFAM mRNA or protein levels in older participants
564 with resistance training. Thus, there are multiple manners to interpret these data. First, training-
565 induced increases in mitochondrial gene expression levels are more so due to mtDNA methylation
566 rather than nuclear DNA hypomethylation (e.g., TFAM). Additionally, while TFAM protein levels
567 did not change with training, this does not exclude the possibility that TFAM binding to the
568 mitochondrial HSP1 and LSP regions increased during various periods throughout the six-week
569 training protocol. Due to tissue limitations, we were not able to perform assays relevant to
570 assessing this phenomenon. However, certain molecular analyses (e.g., ChIP-qPCR or
571 electrophoretic mobility shift assays) can be performed in the future to address this question.
572 There were also interesting correlations that were unveiled (Fig. 2c). As stated prior,
573 methylation of site 106 strongly correlated with knee extensor peak torque (p=0.01) and, to a lesser
574 extent, muscle cross sectional area (p=0.03). Likewise, methylation of position 16455 significantly
575 correlated with both whole-body fat free mass (p=0.02) and vastus lateralis thickness (p=0.03).
576 Given that these are preliminary associations from a limited number of participants, it is difficult
577 to speculate if mtDNA methylation patterns partially or predominantly drive certain phenotypes
578 (e.g., muscle mass, body composition, and/or strength). However, others have found interesting
579 associations with mtDNA methylation patterns in different cell types. For instance, mtDNA
580 bisulphite sequencing results from the blood of 82 individuals aged 18-91 years have shown two
581 CpG sites (M1215 and M1313) located within the 12S ribosomal RNA gene demonstrate an

582 inverse correlation with subject age (47). Others have shown mtDNA copy number is associated
583 with mtDNA methylation levels in tumor-initiating cells (48). Indeed, it is unknown if associations
584 identified herein may be cause-effect in nature, and the relationship between mtDNA methylation
585 patterns and exercise and body composition phenotypes should continue to be explored.
586 While we posit that these are novel and exciting findings for the fields of molecular
587 exercise science and muscle aging biology, various areas need to be further explored. First, this
588 study is limited to predominantly Caucasian males, and future research is needed to address
589 whether the adaptations observed herein are also observed in different races and females. The
590 inclusion of a younger, trained male cohort was for comparative purposes only. However, it is
591 unknown if resistance training can facilitate the same adaptations in this population as well.
592 Training in older participants only spanned 6 weeks, and was two days per week. Although this
593 shorter-term training did elicit changes in various phenotypes according to Table 1, it remains
594 unknown as to whether a greater training frequency and/or long training duration would
595 differentially affect outcomes. Due to tissue limitations, we did not address the mechanism(s)
596 through which mitochondrial demethylation occurred. DNA demethylation (hypomethylation) can
597 occur through the conversion of methylcytosine to hydroxymethylcytosine via ten-eleven
598 translocation (TET) enzymes, and methylation occurs via the de novo methyltransferases
599 (DNMTs) (49). Thus, examining whether resistance training either acutely or chronically
600 downregulates mitochondrial DNMT activity and/or upregulates mitochondrial TET enzyme
601 activity is warranted. It is also notable that a recent review by two of the current co-authors
602 provides evidence to suggest that exercise-induced alterations in muscle metabolites can affect
603 enzymes involved with nuclear (and presumably mitochondrial) DNA hypomethylation (4).
604 Specifically, the authors noted that numerous TCA cycle intermediaries (e.g., FAD/$FADH_2$ ratio,
605 alpha-ketoglutarate, succinate, and fumurate levels) can all influence demethylase activity. Given
606 that the TCA cycle occurs in the mitochondrial matrix, a metabolomics approach in isolated
607 skeletal muscle mitochondria prior to and transiently following a resistance exercise bout could
608 provide clues as to whether metabolic perturbations are associated with some of the methylation
609 patterns observed herein. The assayed mitochondrial markers were also limited in scope. In this
610 regard, markers of mitochondrial function (e.g., state 3 respiration and/or respiratory control ratio
611 (state 3 / state 4) with different substrates, or complex activities) were not examined, and it is
612 possible that some of these markers also coincided with some of the observed molecular
613 adaptations. Moreover, only enough tissue was available to run biochemical assays on crude
614 muscle lysates rather than isolated mitochondria. Given these limitations, it has also not been
615 possible for us to fully understand and explore the impact of small changes in DNA methylation
616 in human mtDNA. While correlations existed between methylation and both phenotypic and
617 transcript measures, fully examining the precise causal role of DNA methylation on these
618 outcomes will be an important development for the field. What also remains to be elucidated is the
619 impact of nutrition on mitochondrial DNA methylation. Indeed, our study was not designed for
620 determining this relationship, and this needs to be further explored. Finally, it is notable that the
621 current study does not provide time course data, and our post-training biopsy (obtained 72 hours
622 following the last exercise bout) may have been too delayed to capture certain methylation events.
623 In this regard, Barres et al. (5) showed that the promoters of various exercise-induced genes were
624 dynamically methylated within a 3-hour post-exercise window in humans. Likewise, this same
625 group reported electrical stimulation elicited dynamic promoter methylation patterns in mouse
626 soleus muscle 45 minutes following contractions. However, it is notable that resistance training
627 dynamically alters gene methylation throughout periods of training, detraining and retraining.

628 Most notably, certain genes show reduced methylation after an acute bout of exercise, and these
629 patterns are retained 22 weeks later (6). Hence, time course data are needed to determine how
630 mtDNA methylation is transiently and chronically affected following exercise stimuli.
631
632 *Conclusions*
633 This is the first study to suggest resistance training in older individuals leads to an appreciable
634 hypomethylation of the mtDNA genome and, specifically, in important regulatory regions.
635 Moreover, observed methylation changes were associated with an increase in various
636 mitochondrial transcripts. Importantly, resistance training restored the mtDNA methylome of older
637 individuals towards profiles observed in younger, trained adults. However, it remains unknown as
638 to whether these events preceded and/or facilitated certain mitochondrial adaptations. What should
639 also be appreciated is that various research has shown that multiple epigenetic alterations (e.g.,
640 histone acetylation) occur with single bouts or longer-term exercise training periods; reviewed in
641 (4). Therefore, relative to the broader epigenetic changes with exercise training, more research is
642 needed to interpret the significance of the current findings.
643
644
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651
652 AUTHOR CONTRIBUTIONS
653 B.A.R., A.P.S., R.A.S., and M.D.R. primarily drafted the manuscript and constructed figures.
654 B.A.R., J.S.G., P.H.C.M., S.C.O., C.G.V., D.A.L., K.C.Y., A.N.K., and M.D.R. were involved in
655 critical aspects of the study in regards to data collection and analyses. D.G.C., S.C.F., A.D.F.
656 provided critical assistance in manuscript preparation. All authors edited the manuscript, and all
657 authors approved the final submitted version.
658
659 DATA AVAILABILITY STATEMENT
660 Several raw data files have been uploaded as supplementary files. Other files can be obtained by
661 emailing the corresponding author (mdr0024@auburn.edu).

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835

836 Table 1. General resistance training adaptations in older males and comparison to younger males

Variable (units)	Mean ± SD	
DXA lean/soft tissue mass (kg)	Older	
	Pre	58.1 ± 5.9$^{\#}$
	Post	58.8 ± 6.2$^{*,\#}$
	Younger, trained	64.9 ± 4.7
VL muscle thickness (cm)	Older	
	Pre	2.09 ± 0.37$^{\#}$
	Post	2.25 ± 0.27$^{*,\#}$
	Younger, trained	3.02±0.35
pQCT mCSA (cm^2)	Older	
	Pre	144.5 ± 20.6$^{\#}$
	Post	148.9 ± 19.5$^{\#}$
	Younger, trained	194.0 ± 22.8
VL peak knee extensor torque (N•m)	Older	
	Pre	144.9 ± 58.0$^{\#}$
	Post	168.7 ± 47.3$^{*,\#}$
	Younger, trained	223.6 ± 27.8

837 Legend: Data are from n=10 older males (age = 65±7 years old) prior to and following six weeks
838 of training as well as basal values in n=7 younger males (22±2 years old) that were resistance-
839 trained (self-reported training age of 5±1 years). All older males were Caucasian, 6/7 younger
840 males were Caucasian, and 1/7 younger males was African American. Abbreviations: DXA,
841 dual-energy x-ray absorptiometry; VL, vastus lateralis. Symbols: *, indicates increase from Pre
842 to Post in older participants (p<0.05); #, indicates different from younger, trained males
843 (p<0.05).

844 Table 2. qPCR primers

Gene	Primer sequences	Amplicon length	Position on gene	NCBI Ref. Seq.
MT-RNR2†	**FP (5′ → 3′):** CGATGGTGCAGCCGCTATTA **RP (5′ → 3′):** ATCATTTACGGGGGAAGGCG	173 bp	**FP:** 3,009-3,028 **RP:** 3,162-3,181	NC_012920
MT-CO1†	**FP (5′ → 3′):** CTTTTCACCGTAGGTGGCCT **RP (5′ → 3′):** AGTGGAAGTGGGCTACAACG	97 bp	**FP:** 6,942-6,961 **RP:** 7,019-7,038	
MT-CYB†	**FP (5′ → 3′):** ACCCCCTAGGAATCACCTCC **RP (5′ → 3′):** GCCTAGGAGGTCTGGTGAGA	134 bp	**FP:** 15,366-15,385 **RP:** 15,480-15,499	
MT-ND5†	**FP (5′ → 3′):** CACATCTGTACCCACGCCTT **RP (5′ → 3′):** AATGCTAGGCTGCCAATGGT	158 bp	**FP:** 13,318-13,337 **RP:** 13,456-13,475	
MT-ND6†	**FP (5′ → 3′):** CCTATTCCCCCGAGCAATCTC **RP (5′ → 3′):** GGAGGATCCTATTGGTGCGG	118 bp	**FP:** 14,149-14,169 **RP:** 14,247-14,266	
TFAM*	**FP (5′ → 3′):** GGCAAGTTGTCCAAAGAAACC **RP (5′ → 3′):** GCATCTGGGTTCTGAGCTTTA	87 bp	**FP:** 276-296 **RP:** 342-362	NM_003201
GAPDH*	**FP (5′ → 3′):** AACCTGCCAAATATGATGAC **RP (5′ → 3′):** TCATACCAGGAAATGAGCTT	193 bp	**FP:** 828-847 **RP:** 1,001-1,020	NM_002046

845 Abbreviations: FP, forward primer; RP, reverse primer; bp, base pairs; MT-RNR2,
846 mitochondrially-encoded 16S rRNA; MT-CO1, cytochrome c oxidase subunit I; MT-CYB,
847 cytochrome B; MT-ND5, NADH dehydrogenase 5; MT-ND6, NADH dehydrogenase 6; TFAM,
848 transcription factor A, mitochondrial; GAPDH, glyceraldehyde-3-phosphate dehydrogenase;
849 Symbols: †, encoded in the mitochondrial genome; *, encoded in the nuclear genome.

850 Figure 1. mtDNA methylome data from older males prior to and following resistance training

851

852 Legend: These data demonstrate read coverage and association with CpG read density (panels A

853 and B). Data is N=10 for older males.

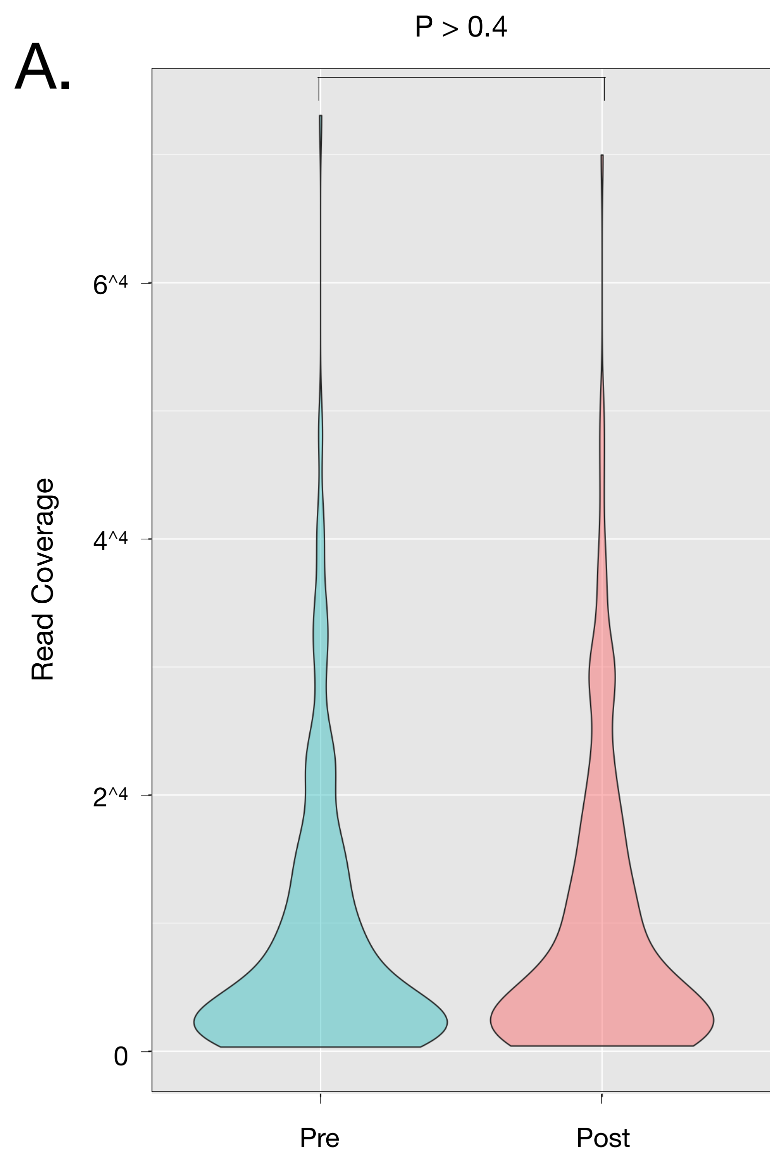
854 Figure 2. Methylation of the mtDNA regulatory region and correlational analyses with
855 phenotypic variables
856
857 Legend: Panel A shows global mitochondrial DNA (mtDNA) methylation decreased following
858 resistance training, and values post-training statistically resembled the younger comparator
859 group. Given that our analyses identified a differentially methylated region (DMR) spanning the
860 known regulatory region of the mtDNA, we further analyzed a 1.12 kb span within this region
861 (panels B and C). Here, we demonstrate that there was significant differential methylation (FDR
862 < 0.05) in older males with six weeks of training within this region (grey shaded region with 4/5
863 CpG sites with difference of >5%). The methylation of CpG sites residing within this locus
864 demonstrated significant correlations with phenotype data (indicated with asterisks) (panel D).
865 Dot coloration represents positive or negative associations, with size and strength of color
866 representing strength of the correlation coefficient. Data is n=10 for all comparisons/correlations
867 of older males, and n=7 for younger trained males. Data for panels B and E are presented as
868 mean ± SEM values.

Figure 3. Mitochondrial transcript and marker adaptations with training in older males

Legend: These data represent mitochondrial transcript levels (panel A), TFAM mRNA levels (panel B), TFAM protein levels (panel C), citrate synthase (CS) activity levels (panel E), and protein levels of mitochondrial complexes I-V (panel F). Panels D and G contain representative Western blots for data in panels C and F, respectively. The color scheme for the legend presented in panel A also applies to panels B/C/E/F. qPCR data contain n=10 older males prior to and following training, and n=7 younger trained males. Citrate synthase activity data contain n=10 older males prior to and following training, and n=6 younger trained males. Western blot contain n=9 older males prior to and following training, and n=6 younger trained males. Symbols: *, indicates increase with training in older males ($p<0.05$); #, indicates different from younger trained males prior to and following training ($p<0.05$). Gene abbreviations can be found in-text. All data are presented as means ± SD values.

882 Figure 4. Correlation between CpG methylation, gene expression and protein abundance in older
883 males prior to and following resistance training
884
885 Legend: Correlation of methylation in CpG sites residing within the 1.12 kb locus of interest in
886 older males prior to and following training shows significant (* = $p<0.05$, ** $p<0.01$)
887 associations between methylation and gene expression (panel A, facet 1). Coloration of dots
888 represents direction of correlation, with strength of color and size of dot representing the strength
889 of correlation coefficients. Highlighted comparisons demonstrated a positive correlation between
890 CpG 16455 methylation and ND5 gene expression ($r=0.57$, $p=0.008$), and an inverse correlation
891 between CpG 162 methylation and 16S rRNA expression ($r = -0.59$, $p=0.005$). Correlation of
892 methylation levels in older males prior to and following training with protein abundance of
893 complexes 3 and 4 yielded significant associations (for all data with relative significance see
894 Suppl. File 4). Of note, CpG sites 163 to 16329 inversely correlated with mtDNA complex 3
895 protein abundance ($p<0.01$), with panel E highlighting the strongest association within (CpG
896 16129; $r = -0.76$, $p<0.001$). Positive correlations between these data sets also existed where, for
897 example, CpG 106 methylation positively correlated with complex 4 (panel D; $r=0.48$, $p=0.044$).
898 Data is $n=10$ for all comparisons/correlations.

A.



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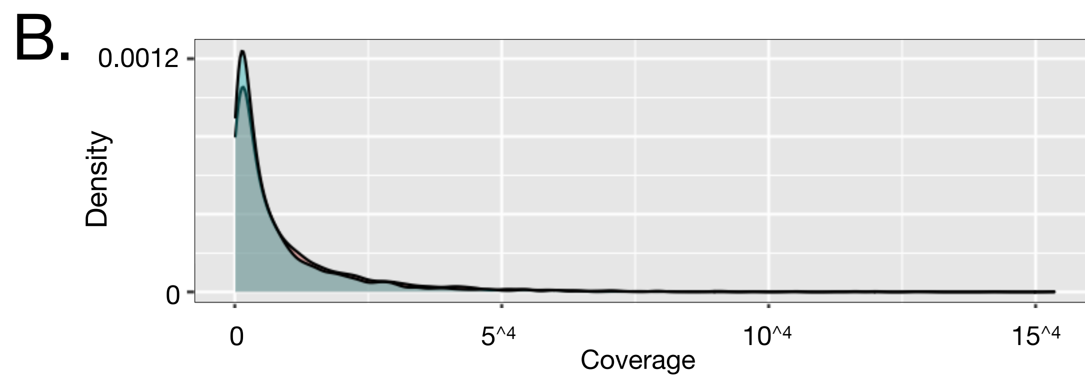
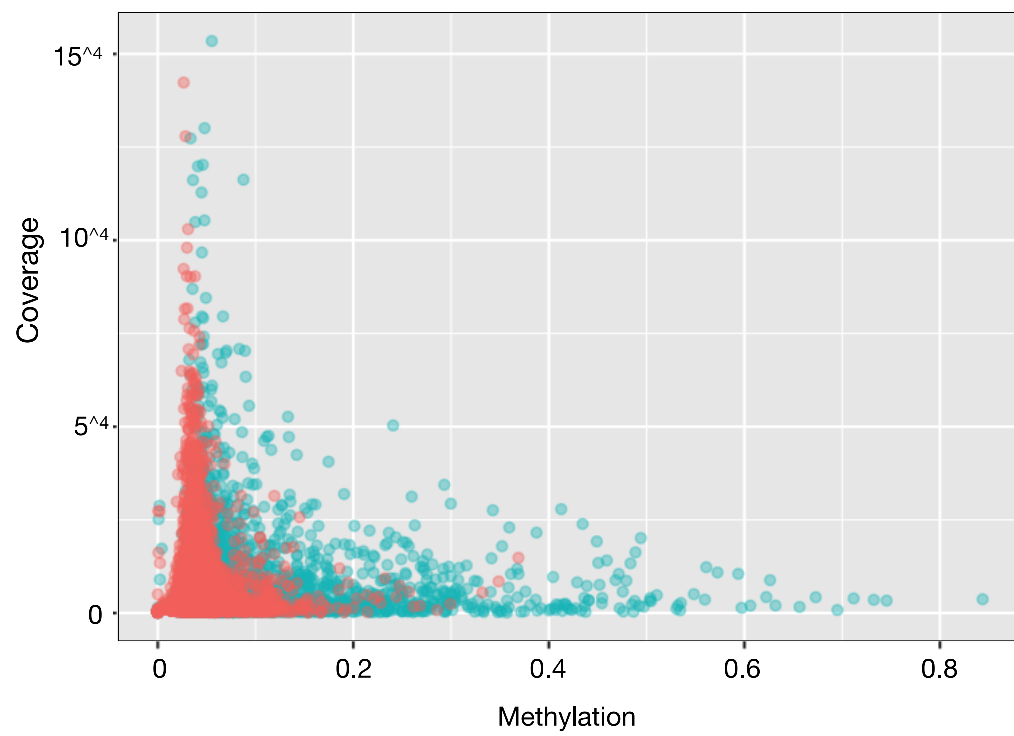
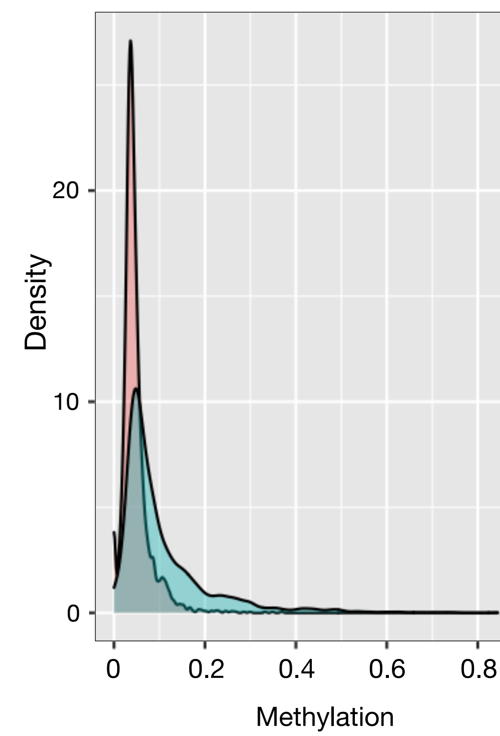
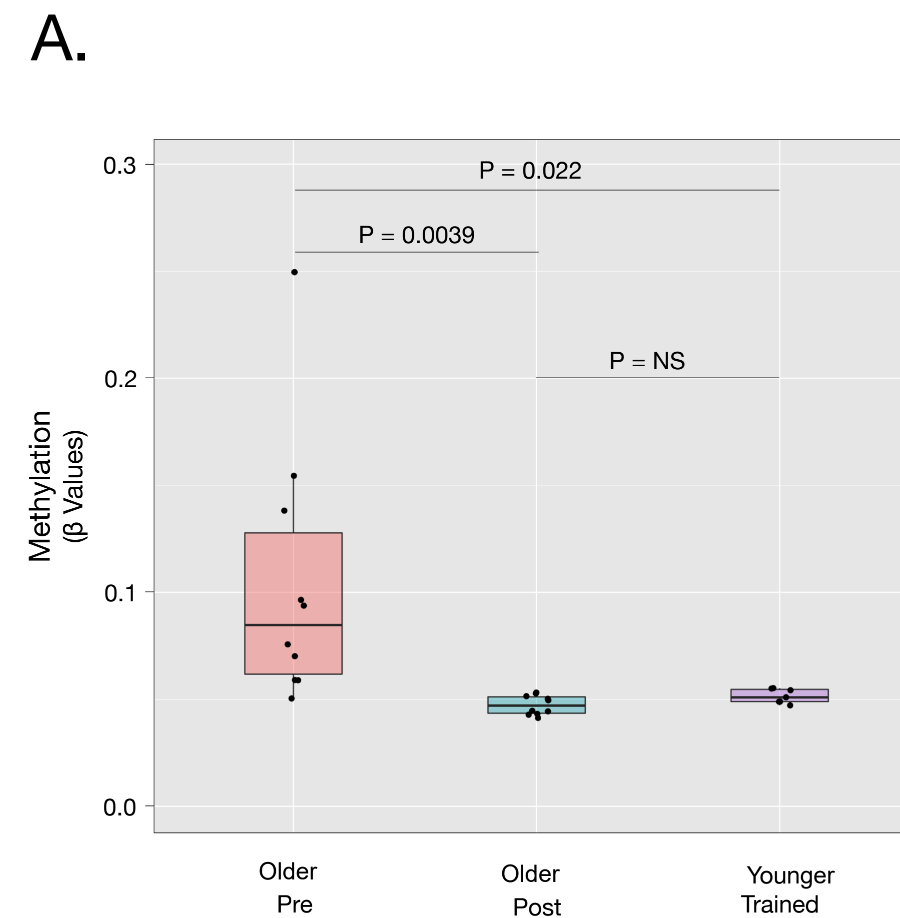
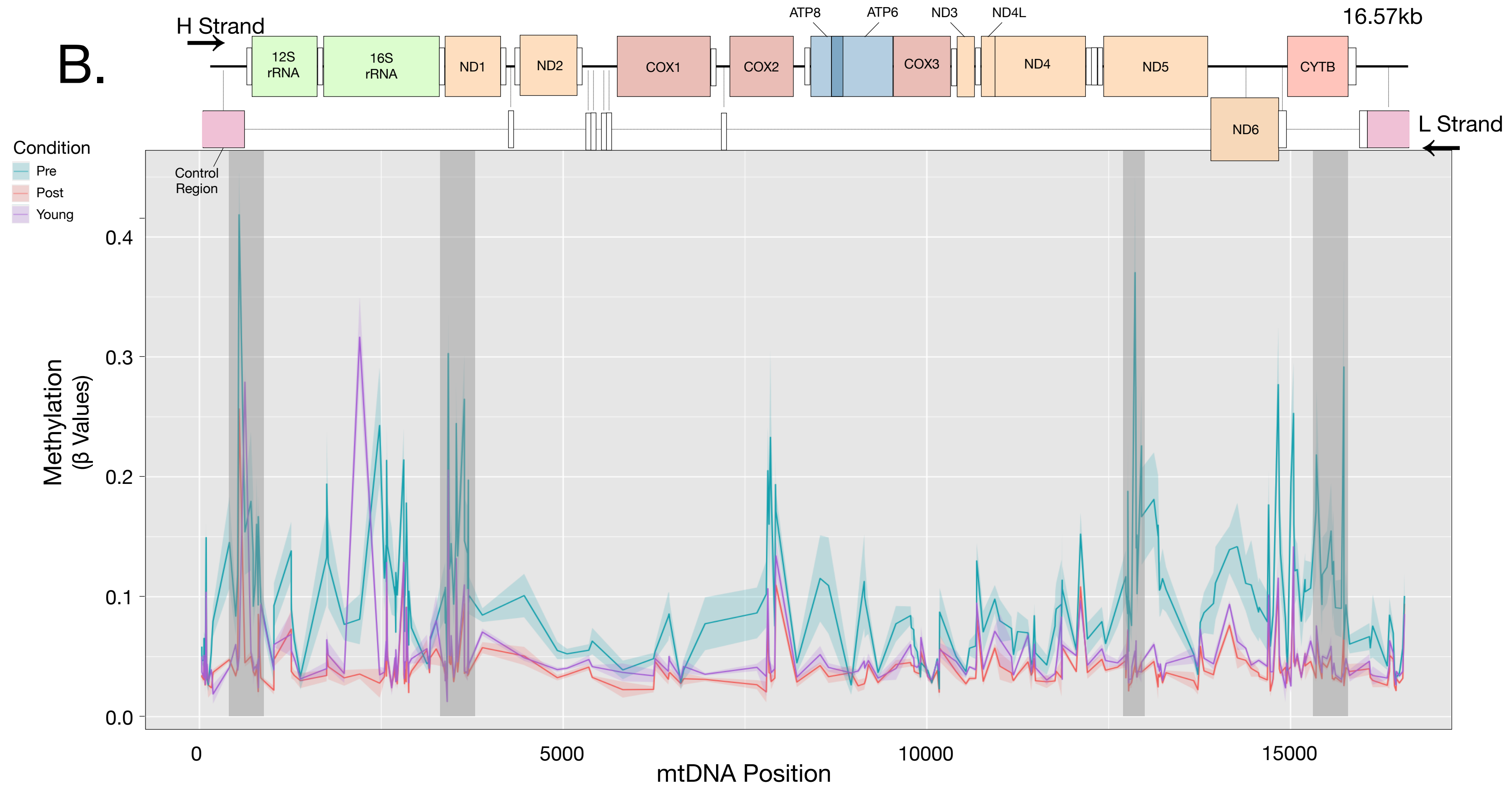
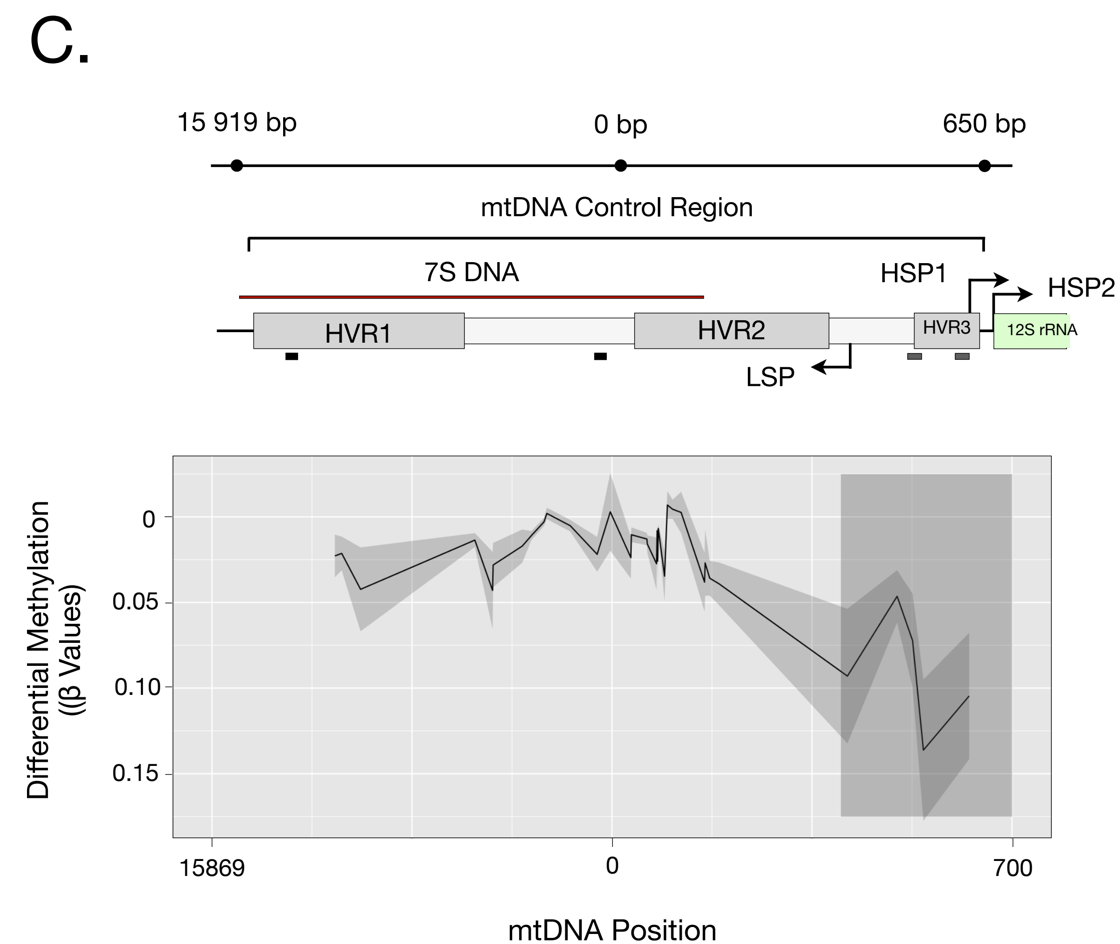


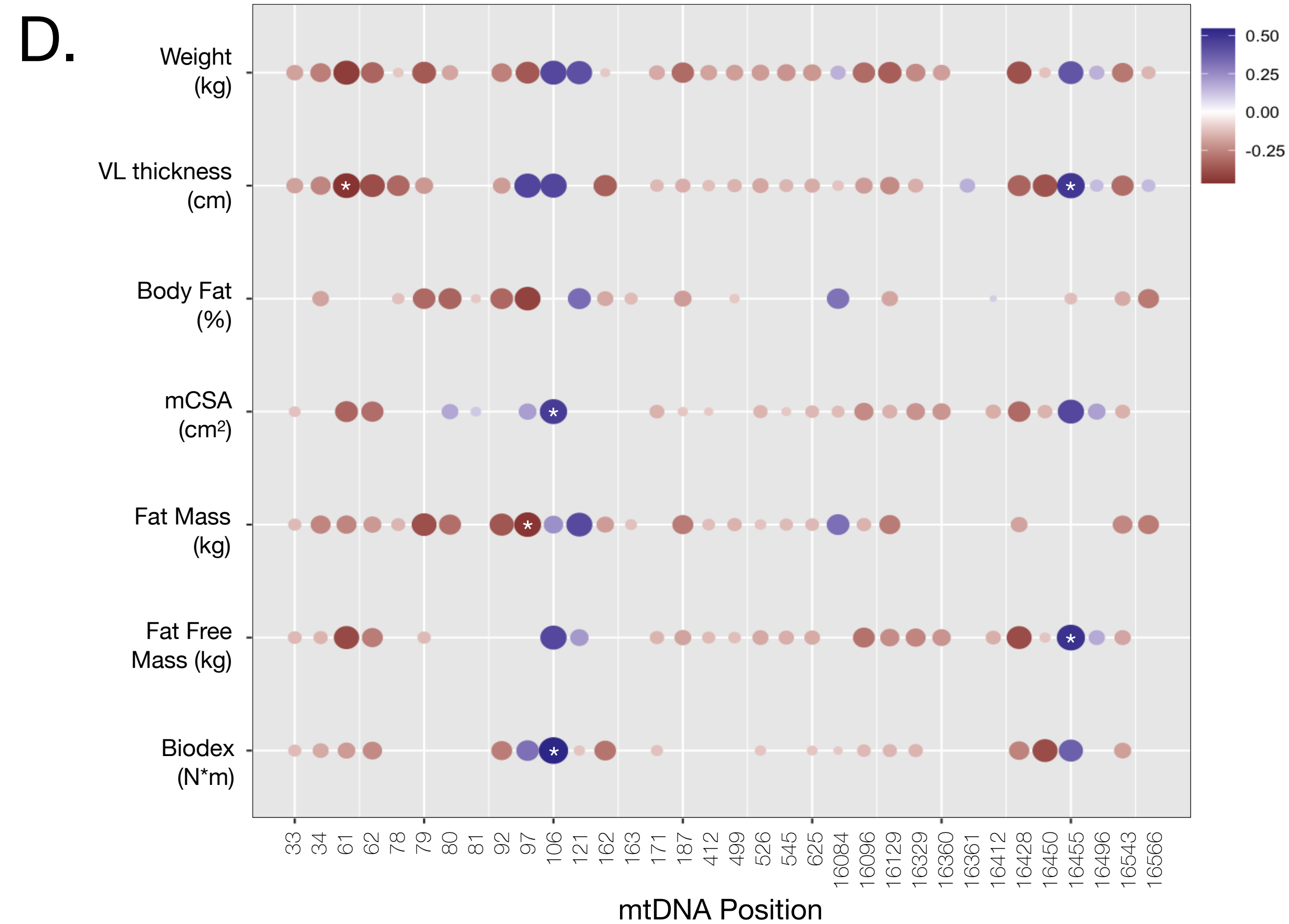


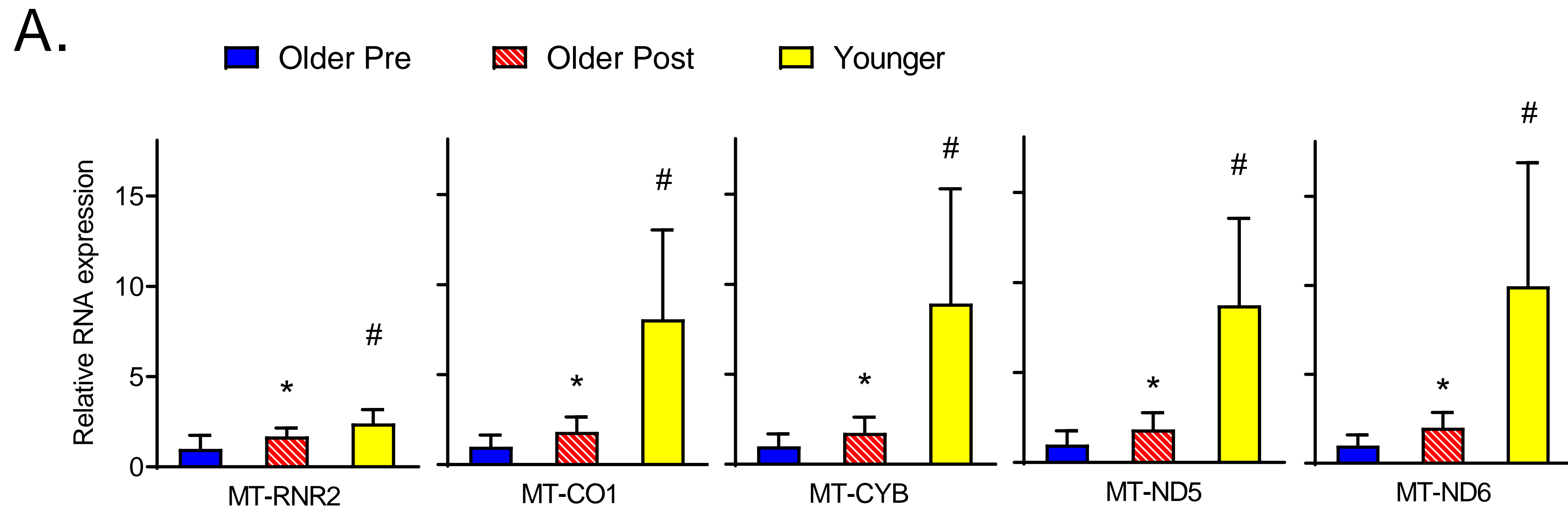


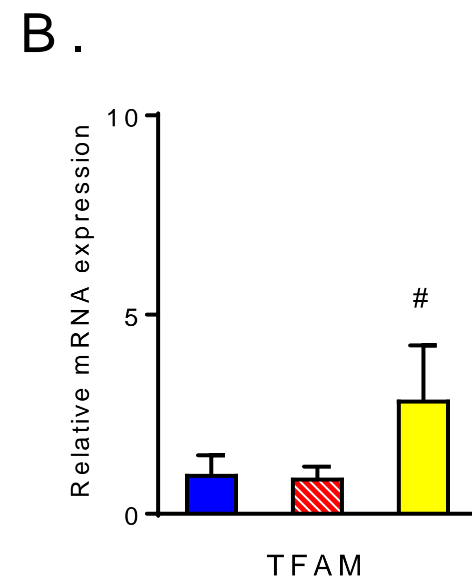

A.
Condition
Pre
Post
Young
P = 0.022
P = 0.0039
P = NS
Methylation
(β Values)
0.3
0.2
0.1
0.0
Older
Pre
Older
Post
Younger
Trained

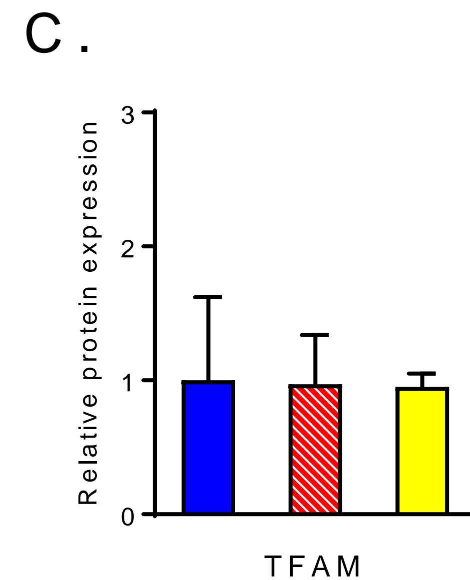

B.
H Strand
16.57kb
12S
rRNA
16S
rRNA
ND1
ND2
COX1
COX2
ATP8
ATP6
COX3
ND3
ND4L
ND4
ND5
CYTB
ND6
L Strand
Control
Region
Methylation
(β Values)
0.4
0.3
0.2
0.1
0.0
0
5000
10000
15000
mtDNA Position

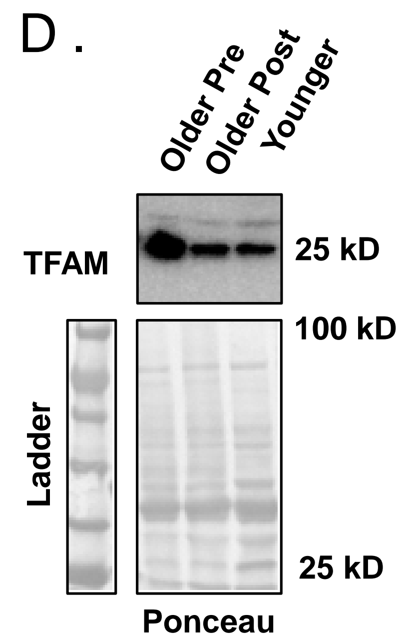

C.
15 919 bp
0 bp
650 bp
mtDNA Control Region
7S DNA
HSP1
HSP2
HVR1
HVR2
HVR3
12S rRNA
LSP
Differential Methylation
(β Values)
0
0.05
0.10
0.15
15869
0
700
mtDNA Position

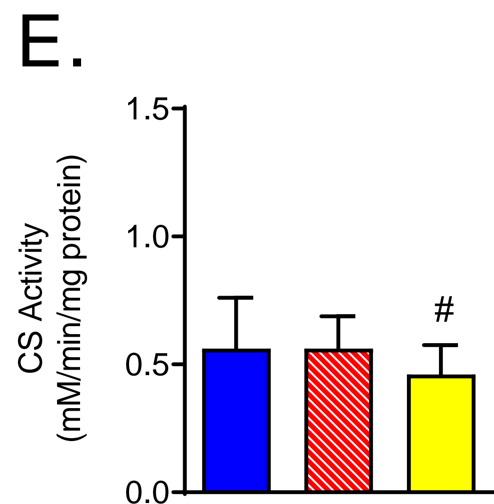

D.
Weight
(kg)
VL thickness
(cm)
Body Fat
(%)
mCSA
(cm²)
Fat Mass
(kg)
Fat Free
Mass (kg)
Biodex
(N*m)
0.50
0.25
0.00
-0.25
33
34
61
62
78
79
80
81
92
97
106
121
162
163
171
187
412
499
526
545
625
16084
16096
16129
16329
16360
16361
16412
16428
16450
16455
16496
16543
16566
mtDNA Position

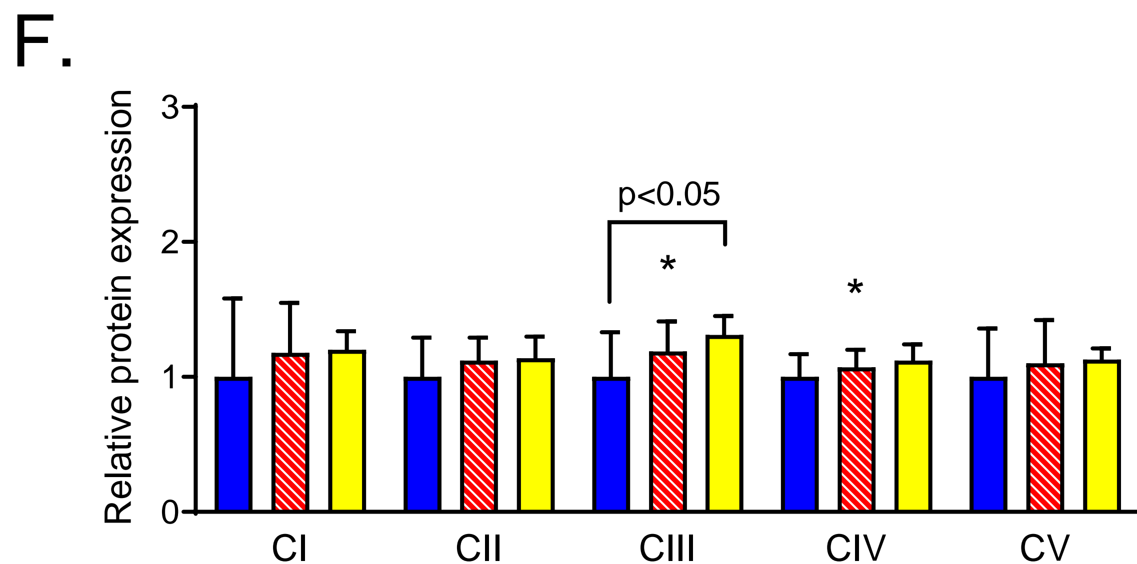

A.
Older Pre
Older Post
Younger
Relative RNA expression
0
5
10
15
MT-RNR2
MT-CO1
MT-CYB
MT-ND5
MT-ND6

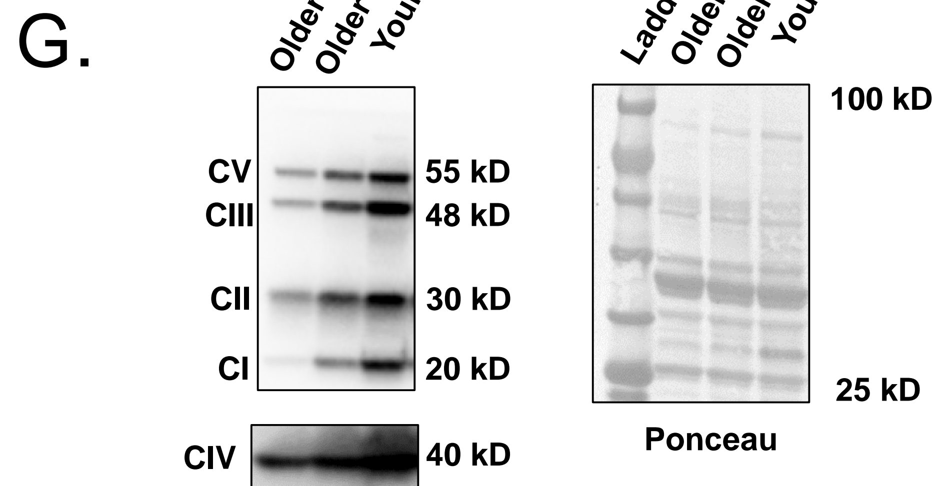

B.
Relative mRNA expression
0
5
10
TFAM

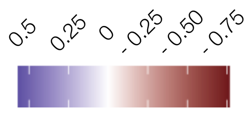

C.
Relative protein expression
0
1
2
3
TFAM


D.
Older Pre
Older Post
Younger
TFAM
25 kD
100 kD
Ladder
25 kD
Ponceau


E.
CS Activity
(mM/min/mg protein)
0.0
0.5
1.0
1.5


F.
Relative protein expression
0
1
2
3
p<0.05
CI
CII
CIII
CIV
CV


G.
Older Pre
Older Post
Younger
CV
CIII
CII
CI
CIV
55 kD
48 kD
30 kD
20 kD
40 kD
Ladder
Older Pre
Older Post
Younger
100 kD
25 kD
Ponceau



A.

ND6 mRNA (fold-change)
ND5 mRNA (fold-change)
CYTB mRNA (fold-change)
COX1 mRNA (fold-change)
16S rRNA mRNA (fold-change)
mito. Complex 4 protein (fold-change)
mito. Complex 3 protein (fold-change)

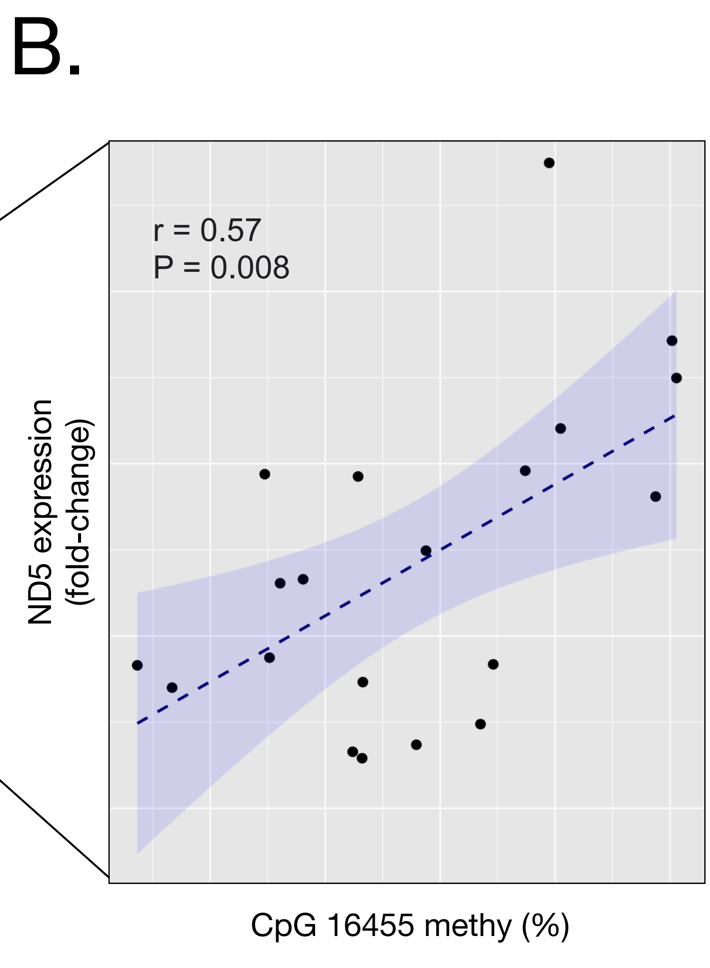
Transcripts
Protein

P < 0.01

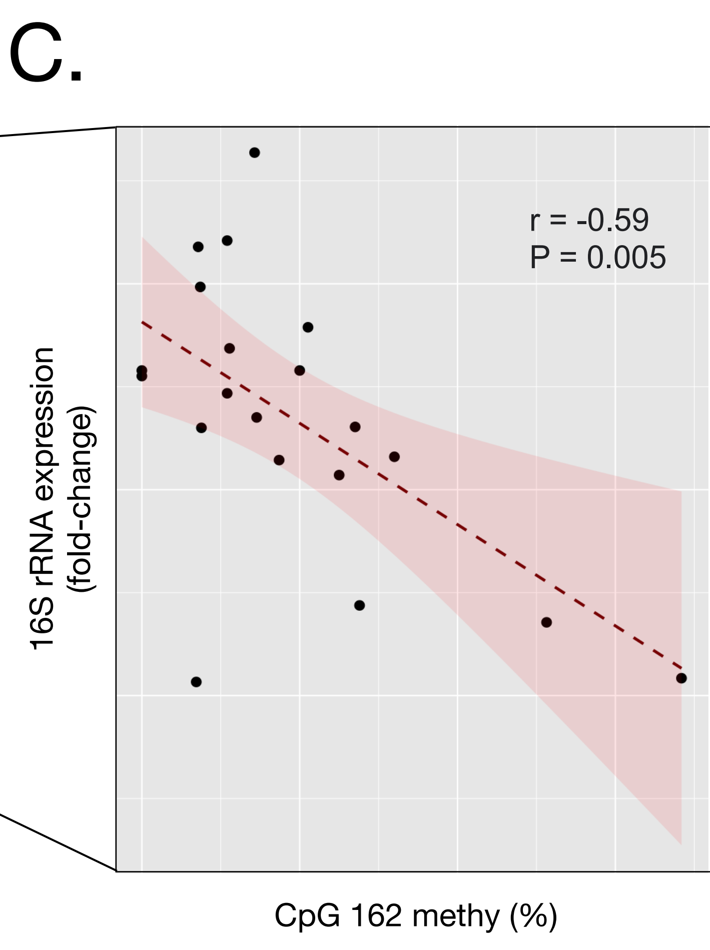
33 34 61 62 78 79 80 81 92 97 106 121 162 163 171 187 412 499 526 545 625 16084 16096 16129 16329 16360 16361 16412 16428 16450 16455 16496 16543 16566

mtDNA Position

B.



C.



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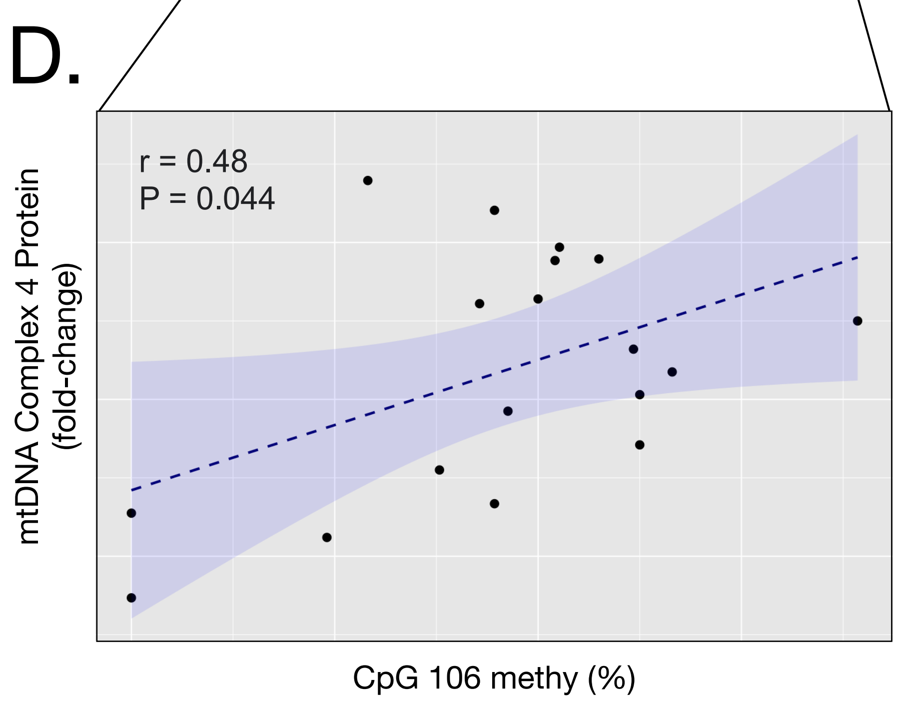

E.