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

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51 ABSTRACT

52 Resistance training (RT) alters skeletal muscle nuclear DNA methylation patterns (or the
53 methylome). However, no study has examined if RT affects the mitochondrial DNA (mtDNA)
54 methylome. Herein, ten older untrained males (65±7 years old) performed six weeks of full-body
55 RT (twice weekly). Body composition and knee extensor torque were assessed prior to and 72
56 hours following the last RT session. Vastus lateralis (VL) biopsies were also obtained. VL DNA
57 was subjected to reduced representation bisulfite sequencing providing excellent coverage across
58 the ~16-kilobase mtDNA methylome (254 CpG sites). Various biochemical assays were also
59 performed, and older male data were compared to younger trained males (22±2 years old, n=7).
60 RT increased whole-body lean tissue mass (p=0.017), VL thickness (p=0.012), and knee extensor
61 torque (p=0.029) in older males. RT also profoundly affected the mtDNA methylome in older
62 males, as 63% (159/254) of the CpG sites demonstrated reduced methylation (p<0.05). Notably,
63 several mtDNA sites presented a more "youthful" signature after RT in older males when
64 comparisons were made to younger males. The 1.12 kilobase D-loop/control region on mtDNA,
65 which regulates mtDNA replication and transcription, possessed enriched hypomethylation in
66 older males following RT. Enhanced expression of mitochondrial H- and L-strand genes and
67 increases in mitochondrial complex III and IV protein levels were also observed (p<0.05). This is
68 the first study to show RT alters the mtDNA methylome in skeletal muscle. Observed methylome
69 alterations may enhance mitochondrial transcription, and RT remarkably evokes mitochondrial
70 methylome profiles to mimic a more youthful signature in older males.

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

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72 INTRODUCTION

73 Resistance training increases strength and muscle mass, and these adaptations have been
74 attributed to various mechanisms (e.g., an increase in satellite cell number, ribosome density, etc.).
75 Critically, molecular adaptations acutely induced by exercise precede well-documented
76 adaptations that occur with chronic training. In this regard, several reports have noted that single
77 exercise bouts transiently orchestrate the up- and down-regulation of hundreds of mRNA
78 transcripts in skeletal muscle (reviewed in (Pillon et al., 2020)). These transcriptional events are
79 complex and involve the coordinated actions of histone-modifying enzymes, transcription factors,
80 transcriptional co-activators, and one of three RNA polymerase enzymes.
81 DNA methylation is a critical mechanism that regulates mRNA transcription (Eden &
82 Cedar, 1994). This process involves a methyl group being transferred to the C-5 position of the
83 cytosine ring, with >98% of methylation occurring at cytosine guanine dinucleotide pairing sites
84 (i.e., CpG sites). DNA methylation is facilitated by DNA methyltransferase (DNMT) enzymes
85 (Jurkowska, Jurkowski, & Jeltsch, 2011), and increased methylation levels in a promoter or
86 enhancer region negatively affect mRNA transcription by either: i) impairing transcription factor
87 binding, and/or ii) compacting DNA and making it transcriptionally inaccessible. Recent
88 enthusiasm has surrounded how exercise alters the collective DNA methylome in skeletal muscle
89 (Seaborne & Sharples, 2020). Barres et al. (Barres et al., 2012) provided the first evidence, at the
90 candidate gene level, to suggest alterations in DNA methylation across canonical metabolic genes
91 in skeletal muscle can occur within hours of a single high-intensity aerobic exercise session.
92 Moreover, the changes in methylation inversely correlated with mRNA expression in the
93 corresponding genes. Subsequently, novel genome-wide methylation (methylome) studies in
94 human skeletal muscle have demonstrated that resistance exercise training (Seaborne, Strauss,
95 Cocks, Shepherd, O'Brien, Someren, et al., 2018; Seaborne, Strauss, Cocks, Shepherd, O'Brien,
96 van Someren, et al., 2018) and acute high intensity running exercise (Maasar et al., 2021) elicit
97 DNA hypomethylation and the upregulation of genes related to actin/cytoskeletal, extracellular
98 matrix, growth-related pathways, and/or metabolic pathways. These same studies have also shown
99 that, following an earlier period of resistance training and detraining, skeletal muscle demonstrates
100 a heightened level of hypomethylation. Importantly, some genes retain a hypomethylated signature
101 following training-induced hypertrophy, even during a period of detraining as muscle mass
102 returned to pre-training levels. Moreover, these genes were 'enhanced' during retraining as a
103 consequence of earlier training, suggesting human skeletal muscle possesses an epigenetic
104 memory of earlier exercise (or 'epi-memory') (Sharples, Stewart, & Seaborne, 2016). The
105 biological process of aging seems to have the opposite effect on the skeletal muscle DNA
106 methylome whereby hypermethylation seemingly accumulates (Turner et al., 2020; S. Voisin et
107 al., 2020; S Voisin et al., 2020; Zykovich et al., 2014). However, increased physical activity
108 (Turner et al., 2020) and resistance exercise (Blocquiaux et al., 2020) have been shown to reverse
109 hypermethylated profiles with age to more hypomethylated signatures.
110 Although resistance training clearly affects molecular mechanisms related to skeletal
111 muscle hypertrophy, the effects of resistance training on mitochondrial adaptations are less clear.
112 Studies in younger adult populations have reported that markers indicative of mitochondrial
113 volume increase, decrease, or do not change in response to several weeks of resistance training
114 (Groennebaek & Vissing, 2017; Parry, Roberts, & Kavazis, 2020). We recently reported that 10
115 weeks of resistance training doubles skeletal muscle citrate synthase activity (a surrogate of
116 mitochondrial volume) in older adults (Lamb, Moore, Mesquita, et al., 2020). Others have also
117 reported that markers reflective of improved mitochondrial function occur in older adults after 14

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118 weeks of resistance training (Parise, Brose, & Tarnopolsky, 2005). Thus, it is plausible that
119 increases in mitochondrial biogenesis and improvements in mitochondrial function may occur in
120 an age-dependent fashion where robust effects are more evident in older versus younger adults.
121 Just as with the nuclear genome, the mitochondrial genome can undergo dynamic DNA
122 methylation and demethylation (Manev, Dzitoyeva, & Chen, 2012). Earlier research in this area
123 suggested alterations in mitochondrial DNA (mtDNA) methylation was relatively low in
124 comparison to the dynamic changes that occur with nuclear DNA methylation (Nass, 1973).
125 Nonetheless, other studies have since suggested that the mtDNA methylome can be transiently
126 modulated through various perturbations. For instance, Wong et al. (Wong, Gertz, Chestnut, &
127 Martin, 2013) used DNA pyrosequencing to demonstrate that mtDNA methylation patterns and
128 mitochondrial DNMT3a levels are abnormal in the skeletal muscle and spinal cord of transgenic
129 mice that develop amyotrophic lateral sclerosis. Moreover, Patil et al. (Patil et al., 2019) recently
130 demonstrated that mtDNA methylation patterns differed in cancerous versus non-cancerous human
131 cell lines. However, given the infancy of this research, it is unclear as to how changes in mtDNA
132 methylation affect mitochondrial physiology.
133 In spite of the tremendous discoveries mentioned above in relation to the nuclear
134 methylome and exercise training, no studies to date have examined how exercise training affects
135 mtDNA methylation patterns in skeletal muscle. This lack of data is, in part, due to methylome
136 studies undertaking array profiling of CpG methylation. Alternatively stated, few human exercise
137 studies have undertaken bisulfite sequencing of skeletal muscle that allows in-depth analysis of
138 mtDNA methylation patterns. Therefore, the current study contained multiple objectives. First, we
139 used a genome-wide DNA bisulfite sequencing strategy (Reduced Representation Bisulfite
140 Sequencing, or RRBS) to determine how six weeks of resistance training altered mtDNA
141 methylation patterns in skeletal muscle of older, previously untrained males. Notably, younger
142 resistance-trained males were also included in this analysis as a comparator group. Next, we
143 determined if the alterations observed at the mtDNA methylome level were associated with
144 corresponding changes in mitochondrial gene expression as well as mitochondrial protein
145 complexes and citrate synthase activity (a marker of mitochondrial volume). Finally, we sought to
146 determine if resistance training was able to rejuvenate the mtDNA methylome profiles of older
147 males to profiles observed in younger males.
148
149
150 RESULTS
151 *Training adaptations*
152 General training adaptations in older males and a comparison to younger males are
153 presented in Table 1. At the end of the six weeks of training, lean/soft tissue mass, vastus lateralis
154 thickness, knee extensor peak torque and mCSA increased in older males ($P = 0.017$, $P = 0.012$, P
155 $= 0.029$, and $P = 0.057$ respectively). However, post-training values in older males were still
156 significantly lower than values in younger, trained males ($P < 0.05$ for all variables).
157
158 INSERT TABLE 1 HERE
159
160 *Mitochondrial DNA methylation*
161 The RRBS data set spanned 254 individual CpG sites mapping to the ~16 kb mtDNA
162 region of the human genome. Comparative analysis in older participants prior to and following
163 resistance training shows that 63% of these CpG sites (159/254) demonstrated a significant

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164 reduction in methylation following training (FDR < 0.05; change ≥ 3%; Suppl. File 1A). Even at
165 a more stringent pre-to-post training change (≥ 5%), a large number of sites (~40%, 98/254 CpGs)
166 possessed a hypomethylated signature. Interestingly, with the same significance criteria, no CpG
167 sites increased in methylation following training. These collective data indicated a globally
168 hypomethylated mtDNA profile following resistance training in older participants (P = 0.0039;
169 Figure 1A). Human mtDNA methylation levels have previously been suggested to be dependent
170 on sequencing coverage biases (Mechta, Ingerslev, Fabre, Picard, & Barres, 2017). We clearly
171 show no differential read coverage issues between time points (Figure 1B), and read density had
172 no association with methylation levels in these conditions (Figure 1C).
173 We subsequently mapped methylation patterns across the 16 kb region and performed
174 differentially methylated region (DMR) analyses to identify loci that were prone to methylation
175 changes following resistance training. Using a 100 bp size sliding window model, with stringent
176 significance thresholds set (e.g. q value < 0.01, differential change of > 5% and with at least 3
177 contiguous 100 bp windows identified), we identified four DMRs (Figure 1D, Suppl. File 1B).
178 Interestingly, this analysis identified a DMR spanning a larger 500 bp region whose origin mapped
179 to that of the D-loop/control region of the human mtDNA genome (Figure 1D).
180
181 INSERT FIGURE 1 HERE
182
183 The entire control region spans a 1.12 kb locus containing a number of extra regulatory elements
184 including the hyper-variable region (HVR), a tertiary DNA fragment (7S DNA), control elements
185 (Mt5 and Mt3L) and TFAM binding sites (Figure 2a). Crucially, the 1.12 kb locus also contains
186 the light strand promotor (LSP), one of the two heavy strand promotors (HSP1), and the HSP2
187 promoter resides less than 100 bp away from this region (Figure 2A). Given that we identified a
188 DMR within this control region (Figure 1D), and this region regulates mtDNA replication and
189 transcription, we examined the locus that spans the mtDNA control region as well as the HSP2
190 region (from 16024 to 650 bp, Figure 2A). In this region, we also identified differentially
191 methylated profiles of the CpGs following resistance training in older participants (Figure 2B).
192 Four of five CpG sites residing within close proximity to either HSP1, HSP2 or LSP showed a
193 significant reduction in methylation (FDR < 0.05, change of > 5%; Figure 2B). This suggests the
194 control region, and in particular the 5'-prime end, is largely hypomethylated following resistance
195 training in older participants.
196 Associating CpG site methylation data in older participants prior to and following training
197 against phenotype variables, we also identified several significant correlations (Figure 2C; Suppl.
198 File 2). CpG sites residing at positions 61 and 97 of the mtDNA genome showed significant inverse
199 correlations between CpG methylation and vastus lateralis thickness as well as whole-body fat
200 mass, respectively (Figure 2C). These same positions also showed association trends between
201 other phenotype variables within our data sets (albeit not significant; Suppl. File 2). Interestingly,
202 two sites (positions 106 and 16455) in our analyses displayed positive correlations between
203 methylation and phenotype variables. Methylation of site 106 strongly correlated with knee
204 extensor peak torque (P = 0.01) and, to a lesser extent, muscle cross sectional area (P = 0.03).
205 Finally, methylation of position 16455 significantly correlated with both whole-body fat free mass
206 (P = 0.02) and vastus lateralis thickness (P = 0.03).
207 Next, we compared the methylation profiles of our older males prior to and following
208 training to younger males to ascertain whether training in the older participants restores mtDNA
209 methylation levels to youth like levels. A significant difference was observed between younger

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210 and older males prior to training ($P = 0.014$). Strikingly, however, methylation patterns of younger
211 participants showed no significant difference compared to older participants following training (P
212 > 0.05). Additionally, across the mtDNA genome, a highly comparable methylation profile existed
213 between younger and older males following training (Figure 2E). Collectively, these data suggest
214 resistance training restored the mtDNA methylome in older participants to mimic a more youthful
215 signature.
216
217 INSERT FIGURE 2 HERE
218
219 *Mitochondrial gene expression and protein complexes*
220 The majority of mtDNA genes are transcribed from the HSP2 region. The primary role of
221 HSP1 is to transcribe mitochondrial rRNA genes on the heavy strand. LSP1 transcribes ND6,
222 which is the only coding transcribing gene on the light strand. We therefore undertook qPCR to
223 assess mitochondrial gene expression of MT-CYB (cytochrome B), MT-ND5 (NADH
224 dehydrogenase 5) and CO1 (cytochrome c oxidase subunit I) as well as MT-RNR2
225 (mitochondrially-encoded 16S rRNA) and light strand ND6 (NADH dehydrogenase 6) mRNA
226 levels. Given TFAM binding sites were located close to the hypomethylated region identified in
227 the DMR analysis above we also measured TFAM gene expression. Resistance training in older
228 participants increased all assayed mitochondrial mRNA targets (MT-CYB, MT-ND6, MT-ND5,
229 and MT-CO1) as well as MT-RNR2 RNA levels ($P < 0.05$) (Figure 3A), but not TFAM expression
230 (Figure 3B; $p > 0.05$). We also confirmed no change in TFAM protein levels ($P > 0.05$; Figure
231 3C). All of the genes analyzed demonstrated higher levels in the younger compared to older males
232 at both the pre- and post-training time points ($P < 0.05$ for all targets). Furthermore, citrate synthase
233 activity assays were performed to assess mitochondrial volume (Figure 3D), and no change
234 occurred in older individuals with resistance training ($P = 1.00$). Skeletal muscle protein levels of
235 electron transport chain complexes were analyzed, and resistance training in older participants
236 increased protein levels of complexes III and IV ($P < 0.05$) (Figure 3E/F).
237
238 INSERT FIGURE 3 HERE
239
240 *Correlation of methylation with mRNA expression and protein levels*
241 We examined associations between CpG methylation patterns and alterations in gene
242 expression to explore the transcriptional consequence of the differentially methylated regions
243 identified. A clear inverse association existed between CpG site methylation (CpG sites residing
244 in our 1.12kb mtDNA loci) and mitochondrial gene expression (Figure 4A). Interestingly, our
245 analysis identified MT-ND6 and MT-RNR2 expression to be the two most commonly inversely
246 associated transcripts (Suppl. Fig 2A and 2B), with CpG site 162 showing the strongest inverse
247 association with MT-RNR2 expression ($r = 0.59$, $P = 0.005$) (Figure 4C). Across all analyses,
248 CpG site 16,329, which resides in HVR1, displayed the most consistent negative/inverse
249 correlation between methylation and gene expression (Suppl. File 3). Counterintuitively, but in
250 keeping with correlational analyses performed on our phenotype data sets, CpG site 16455
251 positively correlated with expression of our analysed gene sets (Figure 4A/B, Suppl. Figure 2A,
252 Suppl. File 3). However, this CpG site is not positioned within any key regulatory locus.
253 We further correlated methylation with the abundance of complex 3 and 4 proteins (Figure
254 4A). Strikingly, methylation of CpG sites 163 to 16329 (Figure A) demonstrated a strong inverse
255 association with complex 3 protein abundance ($P < 0.001$; Figure 4A; Suppl. File 4). CpG site

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256 16129 demonstrated an inverse association (r = -0.76, P = 0.0002; Figure 4E). In keeping with the
257 identification of the positive correlations between CpG methylation of site 106 and phenotypic
258 variables as well as gene expression, we identified a positive correlation (r = 0.48, P = 0.04)
259 between the methylation of this site and complex 4 protein abundance (Figures 4A/D), but such
260 an association was not evident with complex 3 protein abundance.
261
262 INSERT FIGURE 4 HERE
263
264 DISCUSSION
265 This study is the first to illustrate that resistance training leads to a robust hypomethylation
266 of the mitochondrial genome in skeletal muscle. Moreover, resistance training seemingly restored
267 mtDNA methylation signatures in older males relative to younger, trained males. Pre- to post
268 training increases in mitochondrial mRNA and rRNA levels in older participants aligned with the
269 observation that the D loop/control region, which regulates mitochondrial transcription and
270 replication, demonstrated increased hypomethylation with training. There were also interesting
271 associations between mtDNA methylation patterns and various phenotypes. These findings are
272 discussed in greater detail below. From a healthy aging perspective, these data continue to suggest
273 resistance training has beneficial effects on certain aspects of mitochondrial physiology.
274 Skeletal muscle DNA methylation has been reported to increase with aging, and this
275 typically coincides with a decrease in the mRNAs of genes that exist downstream of methylated
276 regions (Blocquiaux et al., 2020; Day et al., 2013; Ling et al., 2007; Ronn et al., 2008; Turner et
277 al., 2020). However, data are lacking in regards to how aging affects the methylation status of the
278 mitochondrial genome in skeletal muscle. D'Aquila et al. (D'Aquila et al., 2015) demonstrated that
279 the methylation of the 12S rRNA region of the mitochondrial genome in PBMCs increases with
280 aging, and 9-year follow-up data illustrate that increased methylation in this region is associated
281 with increased mortality. The current data are in agreement with the findings of D'Aquila and
282 colleagues in that skeletal muscle from older participants prior to training displayed increased
283 mtDNA methylation levels compared to younger, trained participants. Moreover, these
284 methylation patterns coincided with lower mRNA and rRNA levels of various mitochondrial genes
285 as well as lower protein abundances of certain complexes in the older participants. Remarkably,
286 resistance training in older participants decreased mtDNA methylation patterns in certain regions.
287 Although longer-term exercise training has been shown to alter skeletal muscle DNA methylation
288 patterns in younger (Bagley et al., 2020; Seaborne, Strauss, Cocks, Shepherd, O'Brien, Someren,
289 et al., 2018) and middle-aged (Nitert et al., 2012) participants, results from these studies suggest
290 that various genes can be hypo- or hypermethylated. Furthermore, none of these studies
291 interrogated the mtDNA methylation changes given that chip arrays lacking mtDNA probes were
292 utilized. The current findings agree in principle with a recent meta-analysis that examined 16
293 research studies and concluded that nuclear DNA methylation generally decreases with exercise
294 in older adults (Brown, 2015). Additionally, our data agree in principle with other findings that
295 show resistance exercise evokes nuclear genome hypomethylation in older human skeletal muscle
296 (Blocquiaux et al., 2020). However, our findings strongly extend the current literature given that
297 it is the first to suggest exercise training can lead to the hypomethylation of the mitochondrial
298 genome, and DMR analysis demonstrates this hypomethylation is particularly enriched in
299 important regulatory regions for mtDNA transcription and replication.
300 Prior to discussing the implications of the mtDNA methylation data, it is important to
301 appreciate how the nuclear and mitochondrial genomes differ. The nuclear genome contains

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302 approximately 3 billion base pairs, encodes for just over 20,000 genes, and each gene typically
303 contains one segment of DNA separated by regions of non-coding DNA. mtDNA contains 16,569
304 base pairs, and encodes for 37 genes including 2 rRNAs, 22 tRNAs and 13 proteins. The
305 mitochondrial genome possesses a heavy strand (H-strand) and light strand (L-strand) where
306 polycistronic RNAs are transcribed from each strand, and subsequently cleaved and processed to
307 yield mitochondrial rRNAs, tRNAs and mRNAs (Shokolenko & Alexeyev, 2017; Taanman,
308 1999). Two transcription initiation sites exist in a region termed the D-loop. These sites are termed
309 heavy strand promoter 1 (HSP1) and light strand promoter (LSP), and each is where H-strand and
310 L-strand transcription initiation occurs, respectively. Of the mtDNA regions that demonstrated
311 enriched hypomethylation with resistance training in older participants, the most interesting site
312 identified was the D-loop/control region. This finding suggests that resistance training promotes a
313 favorable environment for increased mitochondrial transcription, and aligns with our findings of
314 increased RNA levels H-strand genes (MT-RNR2, MT-CO1, MT-CYB, MT-ND5) and an L-
315 strand gene (MT-ND6) in older individuals following training. While these findings are novel and
316 provocative, it is unclear as to whether the observed methylation and RNA adaptations in older
317 individuals directly facilitated mitochondrial adaptations. In this regard, citrate synthase activity
318 levels, which are strongly associated with mitochondrial volume, remained unaltered with training
319 in older participants. Likewise, only select mitochondrial proteins (specifically, complexes III and
320 IV) were upregulated with training. It is notable, however, that our laboratory has reported 10
321 weeks of resistance training increases mitochondrial biogenesis in older participants (Lamb,
322 Moore, Mesquita, et al., 2020). Moreover, others have reported that longer-term resistance training
323 (~3 months) increases various aspects of mitochondrial function (e.g., respiration and/or complex
324 activities) in older participants (Holloway et al., 2018; Parise et al., 2005; Robinson et al., 2017).
325 Mitochondrial adaptations involve a coordinated effort between the nuclear and mitochondrial
326 genomes given that most mitochondrial proteins are encoded by the nuclear genome (Parry et al.,
327 2020). Thus, the observed mtDNA methylation changes with resistance training may precede
328 certain longer-term mitochondrial adaptations.
329 We were also interested in determining whether TFAM mRNA or protein levels were
330 altered in older participants with resistance training given that TFAM is mitochondrial
331 transcription factor that binds to various regions in the D-loop and is critical for stimulating
332 transcription. There were no alterations in TFAM mRNA or protein levels in older participants
333 with resistance training. However, this does not exclude the possibility that TFAM binding to the
334 mitochondrial HSP1 and LSP regions increased during various periods throughout the six-week
335 training protocol. Due to tissue limitations, we were not able to perform assays relevant to
336 assessing this phenomenon. However, certain molecular analyses (e.g., ChIP-qPCR or
337 electrophoretic mobility shift assays) can be performed in the future to address this question.
338 While we posit that these are novel and exciting findings for the fields of molecular
339 exercise science and muscle aging biology, outstanding questions remain. First, this study is
340 limited to males, and future research is needed to address whether the adaptations observed herein
341 are also observed in females. The inclusion of a younger, trained male cohort was for comparative
342 purposes only. However, it is unknown if resistance training can facilitate the same adaptations in
343 this population as well. Due to tissue limitations, we did not address the mechanism(s) through
344 which mitochondrial demethylation occurred. DNA demethylation (hypomethylation) can occur
345 through the conversion of methylcytosine to hydroxymethylcytosine via ten-eleven translocation
346 (TET) enzymes, and methylation occurs via the de novo methyltransferases (DNMTs) (Wu &
347 Zhang, 2017). Thus, examining whether resistance training either acutely or chronically

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348 downregulates mitochondrial DNMT activity and/or upregulates mitochondrial TET enzyme
349 activity is warranted. It is also notable that a recent review by two of the current co-authors
350 provides evidence to suggest that exercise-induced alterations in muscle metabolites can affect
351 enzymes involved with nuclear (and presumably mitochondrial) DNA hypomethylation (Seaborne
352 & Sharples, 2020). Specifically, the authors noted that numerous TCA cycle intermediaries (e.g.,
353 $FAD/FADH_2$ ratio, alpha-ketoglutarate, succinate, and fumurate levels) can all influence
354 demethylase activity. Given that the TCA cycle occurs in the mitochondrial matrix, a
355 metabolomics approach in isolated skeletal muscle mitochondria prior to and transiently following
356 a resistance exercise bout could provide clues as to whether metabolic perturbations are associated
357 with some of the methylation patterns observed herein. The assayed mitochondrial markers were
358 also limited in scope. In this regard, markers of mitochondrial function (e.g., state III/IV respiration
359 rates with different substrates or complex activities) were not examined, and it is possible that
360 some of these markers also coincided with some of the observed molecular adaptations. Moreover,
361 only enough tissue was available to run biochemical assays on crude muscle lysates rather than
362 isolated mitochondria.
363
364 *Conclusions*
365 This is the first study to suggest resistance training in older individuals leads to an appreciable
366 hypomethylation of the mtDNA genome and, specifically, in important regulatory regions.
367 Moreover, observed methylation changes were associated with an increase in various
368 mitochondrial transcripts. Importantly, resistance training restored the mtDNA methylome of older
369 individuals towards profiles observed in younger, trained adults. However, it remains unknown as
370 to whether these events preceded and/or facilitated certain mitochondrial adaptations. Therefore,
371 more research is needed to interpret the significance of these findings.
372
373
374 EXPERIMENTAL PROCEDURES
375 *Ethical approval*
376 This study was a secondary analysis of two studies approved by the Institutional Review
377 Board at Auburn University. The first protocol (Protocol # 19-249 MR 1907) involved examining
378 the effects of resistance training with daily peanut protein supplementation or no supplementation
379 on skeletal muscle hypertrophy in untrained, older adults between the ages of 50 to 75 years
380 (NCT04015479). Ten older males from the 6-week cohort (n=5 per group) were examined herein.
381 Two-way repeated measures ANOVAs indicated that none of the body composition or assayed
382 biomarkers were affected by peanut protein supplementation (interaction p-values: lean body
383 mass, p=0.952; vastus lateralis (VL) thickness, p=0.543; knee extensor peak torque, p=0.893; all
384 qPCR and Western blot markers, p>0.200; CS activity, p=0.335). The second protocol involved
385 examining the effects of unilateral resistance training on muscle hypertrophy outcomes in seven
386 previously trained young adult males (Protocol # 19-245 MR 1907). Resting baseline biopsies
387 from these participants were used as a comparator group to the older participants to determine if
388 resistance training rejuvenated the mtDNA methylome.
389 Inclusion criteria for both studies required participants to abstain from nutritional
390 supplementation (e.g., creatine monohydrate, protein supplements) one month prior to testing.
391 Participants from both studies had to be free of overt cardio-metabolic diseases (e.g., type II
392 diabetes, severe hypertension, heart failure) or conditions that precluded the collection of a skeletal
393 muscle biopsy. All participants provided verbal and written consent to participate in each

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394 respective study, and both studies conformed to standards set by the latest revision of the
395 Declaration of Helsinki. Data herein included 10 older male participants (age = 65 ± 7 years old;
396 mean ± SD), and 7 previously trained younger adult males (22 ± 2 years old, self-reported
397 resistance training experience of 5 ± 1 years).
398
399 *Resistance training program for older participants*
400 The training program for older males has been previously described (Lamb, Moore, Smith,
401 et al., 2020). Briefly, participants underwent supervised resistance training twice weekly, on non-
402 consecutive days, for six weeks. Each session consisted of five exercises including leg press, leg
403 extensions, lying leg curls, barbell bench press, and cable pull downs. For each exercise,
404 participants performed three sets of 8-12 repetitions to volitional fatigue with at least one minute
405 of rest in between sets. At the end of each set, participants were asked to rate the level of difficulty
406 (0 = easy, 10 = hard). If values were below 7, weight was added to increase effort for the next
407 working set. If values were 10, or the participant could not complete the set, weight was removed
408 prior to the next working set. Participants were encouraged to be as truthful as possible when
409 assessing difficulty. The intent of this training method was to challenge participants where
410 perceived exertion after each set was between a 7-9 rating. This method allowed us to ensure that
411 training effort was maximized within each training session, and that the participants were
412 successfully implementing progressive overload in an individualized fashion.
413
414 *Testing sessions*
415 For younger and older participants, the testing sessions described below occurred during
416 morning hours (05:00–09:00) following an overnight fast. For older males, Pre-testing occurred
417 ~2-5 days prior to the first day of resistance training, and Post-testing occurred 72 hours following
418 the last training bout. The younger males performed all of the same tests described above between
419 05:00-11:00.
420 Prior to testing batteries, participants submitted a urine sample (~5 mL) to assess urine
421 specific gravity (USG) using a handheld refractometer (ATAGO; Bellevue, WA, USA). USG in
422 all participants were <1.020 indicating sufficient hydration (American College of Sports et al.,
423 2007). Height and body mass were assessed using a digital column scale (Seca 769; Hanover, MD,
424 USA), and values were recorded to the nearest 0.1 kg and 0.5 cm, respectively. Participants then
425 had their bone-free lean/soft tissue mass (LSTM) and fat mass determined by a full-body dual-
426 energy x-ray absorptiometry (DXA) scan (Lunar Prodigy; GE Corporation, Fairfield, CT, USA).
427 The same investigator completed all DXA scans. According to previous data published by our
428 laboratory (Kephart et al., 2016), the same-day test-calibrate-retest reliability on 10 participants
429 produced an intra-class correlation coefficient (ICC) of 0.998 for LSTM. After DXA scans, a
430 cross-sectional image of the right thigh at 50% of the femur length was acquired using a peripheral
431 quantitative computed tomography (pQCT) scanner (Stratec XCT 3000, Stratec Medical,
432 Pforzheim, Germany). Scans were acquired using a single 2.4 mm slice thickness, a voxel size of
433 0.4 mm and scanning speed of 20 mm/sec. Images were analyzed for total muscle cross-sectional
434 area (mCSA, cm^2) using the pQCT BoneJ plugin freely available through ImageJ analysis software
435 (NIH, Bethesda, MD, USA). All scans were performed and analyzed by the same investigator, and
436 the ICC was previously determined for mCSA to be 0.990 (*unpublished data*). Following pQCT
437 assessments, right leg vastus lateralis ultrasound assessments were performed using a 3-12 MHz
438 multi-frequency linear phase array transducer (Logiq S7 R2 Expert; General Electric, Fairfield,
439 CT, USA) to determine muscle thickness. Participants were instructed to stand and displace

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440 bodyweight more to the left leg to ensure the right leg was relaxed. Measurements were
441 standardized by placing the transducer at the midway point between the inguinal crease and
442 proximal patella. The same technician performed all ultrasounds. According to previous data from
443 our laboratory, the 24-hour test-retest reliability for muscle thickness assessment on 11 participants
444 resulted in an ICC of 0.983.
445 Right leg vastus lateralis muscle biopsies were then obtained with a 5-gauge needle as
446 previously described (Kephart et al., 2015). Following biopsies, tissue was rapidly teased of blood
447 and connective tissue, wrapped in pre-labeled foils, flash frozen in liquid nitrogen, and
448 subsequently stored at -80°C for further molecular analyses.
449 In older males, right leg knee extensor peak torque testing occurred ~1-3 days prior to the
450 muscle biopsy at the pre time point, whereas this test occurred approximately 30 minutes prior to
451 the biopsy at the post-test time point. In younger males, this test occurred approximately 10
452 minutes following the biopsy. During testing, participants were fastened to an isokinetic
453 dynamometer (Biodex System 4; Biodex Medical Systems, Inc., Shirley, NY, USA). Each
454 participant's knee was aligned with the axis of the dynamometer, and seat height was adjusted to
455 ensure the hip angle was approximately 90°. Prior to peak torque assessment, each participant
456 performed a warmup consisting of submaximal to maximal isokinetic knee extensions. Participants
457 then completed five maximal voluntary isokinetic knee extension actions at 60°/s. Participants
458 were provided verbal encouragement during each contraction. The isokinetic extension resulting
459 in the greatest value for peak torque was used for analyses.
460
461 *Molecular analyses of skeletal muscle.*
462 *DNA isolation.* Muscle samples stored in foils were removed from -80ºC and placed on a
463 liquid nitrogen-cooled ceramic mortar. Tissue was crushed using a ceramic pestle, and ~10 mg
464 was obtained for DNA isolation using the commercially available DNeasy Blood & Tissue Kit
465 (Qiagen; Venlo, The Netherlands; catalog #: 69504) as per the manufacturer's recommendations.
466 DNA pellets were reconstituted in a buffer provided by the kit, and concentrations were determined
467 in duplicate at an absorbance of 260/280 nm (1.81 ± 0.08) using a desktop spectrophotometer
468 (NanoDrop Lite; Thermo Fisher Scientific; Waltham, MA, USA). DNA was then shipped to a
469 commercial vendor (EpiGentek Group Inc.; Farmingdale, NY, USA) for RRBS as described
470 below.
471 *DNA bisulfite conversion and RRBS.* Samples were received by the commercial vendor on
472 dry ice, and were subjected to enzymatic digestion (MSP1 + TaqI); specifically, 300 ng of DNA
473 from each participant was digested for 2 hours with MSP1 enzyme (20U/sample) at 37°C followed
474 by 2 hours with TaqαI (20U/sample) at 65°C. Digested DNA <300 base pair fragments were
475 collected for bisulfite treatment, and bisulfite conversion was performed with the Methylamp DNA
476 Bisulfite Conversion Kit (Epigentek; catalog #: P-1001). The efficiency of bisulfite-treated DNA
477 was determined by real-time PCR using two pairs of primers where the first pair targeted bisulfite-
478 converted beta-actin (BACT), and the second pair targeted unconverted Glyceraldehyde-3-
479 Phosphate Dehydrogenase (GAPDH) for the same bisulfite-treated DNA samples. Library
480 preparation then ensued, and Bioanalyzer QC and KAPA library quantification were performed
481 thereafter. Sample libraries (20 nM) were subjected to multiplex next generation sequencing using
482 an Illumina HiSeq4000 (Illumina Inc.; San Diego, CA, USA). Quality control on raw reads was
483 performed using FASTQC, version 0.11.8
484 (http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc/), and an HTML report was generated for
485 each data set. Quality and adapter trimming was performed on the raw reads using Trim Galore,

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486 version 0.5.0 (http://www.bioinformatics.babraham.ac.uk/projects/trim_galore/). Trim Galore
487 performs the following trimming steps: i) low-quality read removal (Sanger Phred score of 20 or
488 lower), ii) trimming of the 3' Illumina adapter (any signs of AGATCGGAAGAGC), and iii)
489 removal of trimmed reads shorter than 20 bp. Trimmed reads were mapped to the UCSC homo
490 sapiens (human) genome sequence (version GRCh38) using a methylation-aware mapper,
491 bismark, version 0.203.0 (Krueger & Andrews, 2011). Bismark utilizes Bowtie, version 2.2.5
492 (Langmead, Trapnell, Pop, & Salzberg, 2009), with the option "--directional" for targeted bisulfite
493 sequencing libraries and option "--pbat" for post-bisulfite prepared RRBS libraries. For each
494 sample, a summary HTML report was generated, which included alignment and cytosine
495 methylation statistics. Samtools, version 0.1.9 (Li et al., 2009), was utilized to sort the SAM file
496 produced by bismark and remove the duplicate reads due to PCR amplification. Methylation
497 information was extracted from the final bismark mapping result at the base resolution where a
498 minimal read coverage score of 10 and minimal quality score of 20 at each base position are
499 applied. For all comparisons utilized in older adult trained versus untrained data sets, this meant
500 254 CpG sites were covered within the mtDNA. For inclusion of the younger trained adult data
501 set, this number reduced to 253 CpG sites in the mtDNA. The resulting CpG sites were filtered
502 based on coverage and merged for comparative analysis using MethylKit package
503 (https://github.com/al2na/methylKit) in R (version 4.0.3). Only CpG sites that were covered in all
504 participants were merged. Principle Component Analysis plots were subsequently performed to
505 determine group-level quality control. Differentially Methylated Regions (DMRs) and
506 Differentially Methylated CpGs (DMCs) were processed in a similar manner. However, for DMR
507 analysis, data sets were first chunked into 100bp windows with a step size of 100bp. Differential
508 analysis was then performed using MethylKits *calculateDiffmeth* function and logistic regression
509 to calculate differential P values which were then transformed to Q values using the SLIM method
510 (Wang, Tuominen, & Tsai, 2011), and DMRs/DMCs were extracted.
511 *RNA isolation with Trizol and targeted qPCR.* Approximately 10 mg of muscle was placed
512 in 500 µl of Ribozol (Ameresco; Solon, OH, USA), and RNA isolation proceeded following the
513 manufacturer's instructions. RNA concentrations were determined in duplicate using a NanoDrop
514 Lite (Thermo Fisher Scientific), and cDNA (2 µg) was synthesized using a commercial qScript
515 cDNA SuperMix (Quanta Biosciences; Gaithersburg, MD, USA). Real-time qPCR was performed
516 in a thermal cycler (Bio-Rad Laboratories; Hercules, CA, USA) using SYBR green-based methods
517 and gene-specific primers were designed with publically-available software (Primer3Plus;
518 Cambridge, MA, USA). For all primer sets, pilot qPCR reactions and melt curves indicated that
519 only one amplicon was present. The forward and reverse primer sequences of all genes are listed
520 in Table 2. Fold change values were performed using the $2^{\Delta\Delta\text{-}Cq}$ method where $2^{\Delta\text{-}
521 Cq}$ = 2^(housekeeping gene (HKG) Cq - gene of interest Cq), and $2^{\Delta\Delta\text{-}Cq}$ (or fold change) = ($2^{\Delta Cq}$
522 value/$2^{\Delta Cq}$ average of Pre values). GAPDH was used as the reference/HKG gene, and GAPDH
523 Cq values were stable with training in the older participants (pre: 27.85 ± 1.28, post: 27.84 ± 1.25).
524
525 INSERT TABLE 2 HERE
526
527 *Western blotting (mitochondrial complexes and TFAM protein expression).* Muscle stored
528 in foils were removed from -80ºC and placed on a liquid nitrogen-cooled ceramic mortar. Tissue
529 was crushed using a ceramic pestle, and ~20 mg was placed in 1.7 mL microcentrifuge tubes
530 prefilled with general cell lysis buffer (25 mM Tris, pH 7.2, 0.5% Triton X-100, 1x protease
531 inhibitors). Samples were homogenized on ice using hard-plastic pestles, and centrifuged at 1,500

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532 g for 10 minutes at 4°C. Supernatants were collected and placed in new 1.7 mL microtubes on ice.
533 Supernatant protein concentrations were determined using a commercially available BCA kit
534 (Thermo Fisher Scientific; Waltham, MA, USA) according to manufacturer's instructions.
535 Afterwards, supernatants were prepared for Western blotting using 4x Laemmli buffer and distilled
536 water (diH_2O) at a concentration of 1 µg/µL, and denatured for 5 minutes at 100°C prior to being
537 frozen at -80°C until Western blotting. On the day of Western blotting, prepared samples (15 µL)
538 were pipetted onto gradient SDS-polyacrylamide gels (4%–15% Criterion TGX Stain-free gels;
539 Bio-Rad Laboratories), and electrophoresis commenced at 180 V for 50 minutes. Following
540 electrophoresis, proteins were transferred to pre-activated PVDF membranes (Bio-Rad
541 Laboratories) for two hours at 200 mA. Gels were then Ponceau stained for five minutes, washed
542 with diH_2O for one minute, dried for one hour, and digitally imaged with a gel documentation
543 system (ChemiDoc Touch; Bio-Rad Laboratories). Following Ponceau imaging, membranes were
544 re-activated in methanol, blocked with nonfat milk for one hour (5% w/v diluted in Tri-buffered
545 saline with 0.1% Tween 20, or TBST), washed three times in TBST only (5 minutes per wash).
546 Membranes were then incubated for 24 hours with the following antibodies (1:1000 v/v dilution
547 in TBST): i) mouse anti-human OxPhos cocktail (Abcam; Cambridge, MA, USA; catalog#:
548 ab110411), ii) rabbit anti-human TFAM (Abnova; Taipei, Taiwan; catalog #: H00007019-D01P),
549 and iii) COX IV (Cell Signaling Technology; Danvers, MA, USA; Cat# 4850). Following primary
550 antibody incubations, membranes were washed three times in TBST only (5 minutes per wash),
551 and incubated for one hour with horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG
552 (Cell Signaling Technology; catalog #'s: 7076 and 7074). Membranes were then washed three
553 times in TBST only (five minutes per wash), developed using chemiluminescent substrate (EMD
554 Millipore; Burlington, MA, USA), and digitally imaged using a gel documentation system
555 (ChemiDoc Touch; Bio-Rad Laboratories). Raw target band densities were obtained using
556 associated software (Image Lab v6.0.1; Bio-Rad Laboratories), and these values were divided by
557 Ponceau densities at 25-100 kD. Target/Ponceau density ratios were then divided by the grand
558 mean of older participants at the Pre time point in order to obtain relative protein expression values.
559 For Western blotting, 9 of 10 older participants and 6 of 7 younger males were assayed due to
560 tissue limitations.
561 *Determination of muscle citrate synthase activity.* Muscle citrate synthase activity levels
562 were determined in duplicate on supernatants obtained from muscle described in the Western
563 blotting section; notably, these methods are similar to previous methods used by our laboratory
564 (Haun et al., 2019; Roberts et al., 2018). The assay principle is based on the reduction of 5,50-
565 dithiobis(2- nitrobenzoic acid) (DTNB) at 412 nm (extinction coefficient 13.6 mmol/L/cm)
566 coupled to the reduction of acetyl-CoA by the citrate synthase reaction in the presence of
567 oxaloacetate. Briefly, 12.5 µg of skeletal muscle protein obtained from supernatants was added to
568 a mixture composed of 0.125 mol/L Tris–HCl (pH 8.0), 0.03 mmol/L acetyl-CoA, and 0.1 mmol/L
569 DTNB. All duplicate reactions occurred in 96-well plates, reactions were initiated by the addition
570 of 5 µL of 50 mmol/L oxaloacetate per well, and the absorbance change was recorded for 60
571 seconds in a spectrophotometer (Synergy H1; BioTek; Winooski, VT, USA). Again, 6 of 7
572 younger males were assayed due to tissue limitations.
573
574 *Statistics*
575 Phenotype and select molecular data were compared from pre to post training in older
576 males using dependent samples t-tests. Additionally, comparisons of data from older males at these
577 time points were made to younger males using independent samples t-tests. Methylation data were

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578 analyzed from pre- to post-training in older males and between older and younger participants
579 using a variety of statistical methods that are described in greater detail in the results section.
580 Select dependent variables were also correlated using Pearson's correlation coefficients. All data
581 herein are presented in figures and tables as means ± standard deviation values unless stated
582 otherwise, and statistical significance was set at $p < 0.05$.
583
584

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590
591

592 CONFLICT OF INTEREST STATEMENT
593 None of the authors have financial or other conflicts of interest to report with regard to these data.
594
595

596 AUTHOR CONTRIBUTIONS
597 B.A.R., A.P.S., R.A.S., and M.D.R. primarily drafted the manuscript and constructed figures.
598 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
599 critical aspects of the study in regards to data collection and analyses. D.G.C., S.C.F., A.D.F.
600 provided critical assistance in manuscript preparation. All authors edited the manuscript, and all
601 authors approved the final submitted version.
602
603

604 DATA AVAILABILITY STATEMENT
605 Several raw data files have been uploaded as supplementary files. Other files can be obtained by
606 emailing the corresponding author (mdr0024@auburn.edu).

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607 REFERENCES

608

609 American College of Sports, M., Sawka, M. N., Burke, L. M., Eichner, E. R., Maughan, R. J.,
610 Montain, S. J., & Stachenfeld, N. S. (2007). American College of Sports Medicine
611 position stand. Exercise and fluid replacement. *Med Sci Sports Exerc, 39*(2), 377-390.
612 doi:10.1249/mss.0b013e31802ca597
613 Bagley, J. R., Burghardt, K. J., McManus, R., Howlett, B., Costa, P. B., Coburn, J. W., . . .
614 Galpin, A. J. (2020). Epigenetic Responses to Acute Resistance Exercise in Trained vs.
615 Sedentary Men. *J Strength Cond Res, 34*(6), 1574-1580.
616 doi:10.1519/JSC.0000000000003185
617 Barres, R., Yan, J., Egan, B., Treebak, J. T., Rasmussen, M., Fritz, T., . . . Zierath, J. R. (2012).
618 Acute exercise remodels promoter methylation in human skeletal muscle. *Cell Metab,*
619 *15*(3), 405-411. doi:10.1016/j.cmet.2012.01.001
620 Blocquiaux, S., Ramaekers, M., Van Thienen, R., Nielens, H., Delecluse, C., De Bock, K., &
621 Thomis, M. (2020). Recurrent training rejuvenates and enhances transcriptome and
622 methylome responses in young and older human muscle. *bioRxiv*,
623 2020.2006.2030.179465. doi:10.1101/2020.06.30.179465
624 Brown, W. M. (2015). Exercise-associated DNA methylation change in skeletal muscle and the
625 importance of imprinted genes: a bioinformatics meta-analysis. *Br J Sports Med, 49*(24),
626 1567-1578. doi:10.1136/bjsports-2014-094073
627 D'Aquila, P., Giordano, M., Montesanto, A., De Rango, F., Passarino, G., & Bellizzi, D. (2015).
628 Age-and gender-related pattern of methylation in the MT-RNR1 gene. *Epigenomics, 7*(5),
629 707-716. doi:10.2217/epi.15.30
630 Day, K., Waite, L. L., Thalacker-Mercer, A., West, A., Bamman, M. M., Brooks, J. D., . . .
631 Absher, D. (2013). Differential DNA methylation with age displays both common and
632 dynamic features across human tissues that are influenced by CpG landscape. *Genome*
633 *Biol, 14*(9), R102. doi:10.1186/gb-2013-14-9-r102
634 Eden, S., & Cedar, H. (1994). Role of DNA methylation in the regulation of transcription. *Curr*
635 *Opin Genet Dev, 4*(2), 255-259. doi:10.1016/s0959-437x(05)80052-8
636 Groennebaek, T., & Vissing, K. (2017). Impact of Resistance Training on Skeletal Muscle
637 Mitochondrial Biogenesis, Content, and Function. *Front Physiol, 8*, 713.
638 doi:10.3389/fphys.2017.00713
639 Haun, C. T., Vann, C. G., Osburn, S. C., Mumford, P. W., Roberson, P. A., Romero, M. A., . . .
640 Roberts, M. D. (2019). Muscle fiber hypertrophy in response to 6 weeks of high-volume
641 resistance training in trained young men is largely attributed to sarcoplasmic hypertrophy.
642 *PLoS One, 14*(6), e0215267. doi:10.1371/journal.pone.0215267
643 Holloway, G. P., Holwerda, A. M., Miotto, P. M., Dirks, M. L., Verdijk, L. B., & van Loon, L. J.
644 C. (2018). Age-Associated Impairments in Mitochondrial ADP Sensitivity Contribute to
645 Redox Stress in Senescent Human Skeletal Muscle. *Cell Rep, 22*(11), 2837-2848.
646 doi:10.1016/j.celrep.2018.02.069
647 Jurkowska, R. Z., Jurkowski, T. P., & Jeltsch, A. (2011). Structure and function of mammalian
648 DNA methyltransferases. *Chembiochem, 12*(2), 206-222. doi:10.1002/cbic.201000195
649 Kephart, W. C., Mobley, C. B., Fox, C. D., Pascoe, D. D., Sefton, J. M., Wilson, T. J., . . .
650 Martin, J. S. (2015). A single bout of whole-leg, peristaltic pulse external pneumatic
651 compression upregulates PGC-1alpha mRNA and endothelial nitric oxide sythase protein
652 in human skeletal muscle tissue. *Exp Physiol, 100*(7), 852-864. doi:10.1113/EP085160

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653 Kephart, W. C., Wachs, T. D., Thompson, R. M., Brooks Mobley, C., Fox, C. D., McDonald, J.
654 R., . . . Roberts, M. D. (2016). Ten weeks of branched-chain amino acid supplementation
655 improves select performance and immunological variables in trained cyclists. *Amino*
656 *Acids, 48*(3), 779-789. doi:10.1007/s00726-015-2125-8
657 Krueger, F., & Andrews, S. R. (2011). Bismark: a flexible aligner and methylation caller for
658 Bisulfite-Seq applications. *Bioinformatics, 27*(11), 1571-1572.
659 doi:10.1093/bioinformatics/btr167
660 Lamb, D. A., Moore, J. H., Mesquita, P. H. C., Smith, M. A., Vann, C. G., Osburn, S. C., . . .
661 Roberts, M. D. (2020). Resistance training increases muscle NAD(+) and NADH
662 concentrations as well as NAMPT protein levels and global sirtuin activity in middle-
663 aged, overweight, untrained individuals. *Aging (Albany NY), 12*(10), 9447-9460.
664 doi:10.18632/aging.103218
665 Lamb, D. A., Moore, J. H., Smith, M. A., Vann, C. G., Osburn, S. C., Ruple, B. A., . . . Roberts,
666 M. D. (2020). The effects of resistance training with or without peanut protein
667 supplementation on skeletal muscle and strength adaptations in older individuals. *J Int*
668 *Soc Sports Nutr, 17*(1), 66. doi:10.1186/s12970-020-00397-y
669 Langmead, B., Trapnell, C., Pop, M., & Salzberg, S. L. (2009). Ultrafast and memory-efficient
670 alignment of short DNA sequences to the human genome. *Genome Biol, 10*(3), R25.
671 doi:10.1186/gb-2009-10-3-r25
672 Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., . . . Genome Project Data
673 Processing, S. (2009). The Sequence Alignment/Map format and SAMtools.
674 *Bioinformatics, 25*(16), 2078-2079. doi:10.1093/bioinformatics/btp352
675 Ling, C., Poulsen, P., Simonsson, S., Ronn, T., Holmkvist, J., Almgren, P., . . . Groop, L. (2007).
676 Genetic and epigenetic factors are associated with expression of respiratory chain
677 component NDUFB6 in human skeletal muscle. *J Clin Invest, 117*(11), 3427-3435.
678 doi:10.1172/JCI30938
679 Maasar, M. F., Turner, D. C., Gorski, P. P., Seaborne, R. A., Strauss, J. A., Shepherd, S. O., . . .
680 Sharples, A. P. (2021). The Comparative Methylome and Transcriptome After Change of
681 Direction Compared to Straight Line Running Exercise in Human Skeletal Muscle. *Front*
682 *Physiol, 12*, 619447. doi:10.3389/fphys.2021.619447
683 Manev, H., Dzitoyeva, S., & Chen, H. (2012). Mitochondrial DNA: A Blind Spot in
684 Neuroepigenetics. *Biomol Concepts, 3*(2), 107-115. doi:10.1515/bmc-2011-0058
685 Mechta, M., Ingerslev, L. R., Fabre, O., Picard, M., & Barres, R. (2017). Evidence Suggesting
686 Absence of Mitochondrial DNA Methylation. *Front Genet, 8*, 166.
687 doi:10.3389/fgene.2017.00166
688 Nass, M. M. (1973). Differential methylation of mitochondrial and nuclear DNA in cultured
689 mouse, hamster and virus-transformed hamster cells. In vivo and in vitro methylation. *J*
690 *Mol Biol, 80*(1), 155-175. doi:10.1016/0022-2836(73)90239-8
691 Nitert, M. D., Dayeh, T., Volkov, P., Elgzyri, T., Hall, E., Nilsson, E., . . . Ling, C. (2012).
692 Impact of an exercise intervention on DNA methylation in skeletal muscle from first-
693 degree relatives of patients with type 2 diabetes. *Diabetes, 61*(12), 3322-3332.
694 doi:10.2337/db11-1653
695 Parise, G., Brose, A. N., & Tarnopolsky, M. A. (2005). Resistance exercise training decreases
696 oxidative damage to DNA and increases cytochrome oxidase activity in older adults. *Exp*
697 *Gerontol, 40*(3), 173-180. doi:10.1016/j.exger.2004.09.002

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698 Parry, H. A., Roberts, M. D., & Kavazis, A. N. (2020). Human Skeletal Muscle Mitochondrial
699 Adaptations Following Resistance Exercise Training. *Int J Sports Med, 41*(6), 349-359.
700 doi:10.1055/a-1121-7851
701 Patil, V., Cuenin, C., Chung, F., Aguilera, J. R. R., Fernandez-Jimenez, N., Romero-Garmendia,
702 I., . . . Herceg, Z. (2019). Human mitochondrial DNA is extensively methylated in a non-
703 CpG context. *Nucleic Acids Res, 47*(19), 10072-10085. doi:10.1093/nar/gkz762
704 Pillon, N. J., Gabriel, B. M., Dollet, L., Smith, J. A. B., Sardon Puig, L., Botella, J., . . . Zierath,
705 J. R. (2020). Transcriptomic profiling of skeletal muscle adaptations to exercise and
706 inactivity. *Nat Commun, 11*(1), 470. doi:10.1038/s41467-019-13869-w
707 Roberts, M. D., Romero, M. A., Mobley, C. B., Mumford, P. W., Roberson, P. A., Haun, C. T., .
708 . . Kavazis, A. N. (2018). Skeletal muscle mitochondrial volume and myozenin-1 protein
709 differences exist between high versus low anabolic responders to resistance training.
710 *PeerJ, 6*, e5338. doi:10.7717/peerj.5338
711 Robinson, M. M., Dasari, S., Konopka, A. R., Johnson, M. L., Manjunatha, S., Esponda, R. R., . .
712 . Nair, K. S. (2017). Enhanced Protein Translation Underlies Improved Metabolic and
713 Physical Adaptations to Different Exercise Training Modes in Young and Old Humans.
714 *Cell Metab, 25*(3), 581-592. doi:10.1016/j.cmet.2017.02.009
715 Ronn, T., Poulsen, P., Hansson, O., Holmkvist, J., Almgren, P., Nilsson, P., . . . Ling, C. (2008).
716 Age influences DNA methylation and gene expression of COX7A1 in human skeletal
717 muscle. *Diabetologia, 51*(7), 1159-1168. doi:10.1007/s00125-008-1018-8
718 Seaborne, R. A., & Sharples, A. P. (2020). The Interplay Between Exercise Metabolism,
719 Epigenetics, and Skeletal Muscle Remodeling. *Exerc Sport Sci Rev, 48*(4), 188-200.
720 doi:10.1249/JES.0000000000000227
721 Seaborne, R. A., Strauss, J., Cocks, M., Shepherd, S., O'Brien, T. D., Someren, K. A. V., . . .
722 Sharples, A. P. (2018). Methylome of human skeletal muscle after acute & chronic
723 resistance exercise training, detraining & retraining. *Sci Data, 5*, 180213.
724 doi:10.1038/sdata.2018.213
725 Seaborne, R. A., Strauss, J., Cocks, M., Shepherd, S., O'Brien, T. D., van Someren, K. A., . . .
726 Sharples, A. P. (2018). Human Skeletal Muscle Possesses an Epigenetic Memory of
727 Hypertrophy. *Sci Rep, 8*(1), 1898. doi:10.1038/s41598-018-20287-3
728 Sharples, A. P., Stewart, C. E., & Seaborne, R. A. (2016). Does skeletal muscle have an 'epi'-
729 memory? The role of epigenetics in nutritional programming, metabolic disease, aging
730 and exercise. *Aging Cell, 15*(4), 603-616. doi:10.1111/acel.12486
731 Shokolenko, I. N., & Alexeyev, M. F. (2017). Mitochondrial transcription in mammalian cells.
732 *Front Biosci (Landmark Ed), 22*, 835-853. doi:10.2741/4520
733 Taanman, J. W. (1999). The mitochondrial genome: structure, transcription, translation and
734 replication. *Biochim Biophys Acta, 1410*(2), 103-123. doi:10.1016/s0005-2728(98)00161-
735 3
736 Turner, D. C., Gorski, P. P., Maasar, M. F., Seaborne, R. A., Baumert, P., Brown, A. D., . . .
737 Sharples, A. P. (2020). DNA methylation across the genome in aged human skeletal
738 muscle tissue and muscle-derived cells: the role of HOX genes and physical activity. *Sci*
739 *Rep, 10*(1), 15360. doi:10.1038/s41598-020-72730-z
740 Voisin, S., Harvey, N. R., Haupt, L. M., Griffiths, L. R., Ashton, K. J., Coffey, V. G., . . . Eynon,
741 N. (2020). An epigenetic clock for human skeletal muscle. *J Cachexia Sarcopenia*
742 *Muscle, 11*(4), 887-898. doi:10.1002/jcsm.12556

bioRxiv preprint doi: https://doi.org/10.1101/2021.04.13.439651; this version posted April 13, 2021. The copyright holder for this preprint (which was not certified by peer review) is the author/funder, who has granted bioRxiv a license to display the preprint in perpetuity. It is made available under aCC-BY 4.0 International license.

743 Voisin, S., Jacques, M., Landen, S., Harvey, N., Haupt, L., Griffiths, L., . . . Eynon, N. (2020).
744 Meta-analysis of genome-wide DNA methylation and integrative OMICs in human
745 skeletal muscle. *bioRxiv*, 2020.2009.2028.315838. doi:10.1101/2020.09.28.315838
746 Wang, H. Q., Tuominen, L. K., & Tsai, C. J. (2011). SLIM: a sliding linear model for estimating
747 the proportion of true null hypotheses in datasets with dependence structures.
748 *Bioinformatics, 27*(2), 225-231. doi:10.1093/bioinformatics/btq650
749 Wong, M., Gertz, B., Chestnut, B. A., & Martin, L. J. (2013). Mitochondrial DNMT3A and
750 DNA methylation in skeletal muscle and CNS of transgenic mouse models of ALS. *Front*
751 *Cell Neurosci, 7*, 279. doi:10.3389/fncel.2013.00279
752 Wu, X., & Zhang, Y. (2017). TET-mediated active DNA demethylation: mechanism, function
753 and beyond. *Nat Rev Genet, 18*(9), 517-534. doi:10.1038/nrg.2017.33
754 Zykovich, A., Hubbard, A., Flynn, J. M., Tarnopolsky, M., Fraga, M. F., Kerksick, C., . . .
755 Melov, S. (2014). Genome-wide DNA methylation changes with age in disease-free
756 human skeletal muscle. *Aging Cell, 13*(2), 360-366. doi:10.1111/acel.12180

757

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758 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

759 Legend: Data are from n=10 older males (age = 65±7 years old) prior to and following six weeks
760 of training as well as basal values in n=7 younger males (22±2 years old) that were resistance-
761 trained (self-reported training age of 5±1 years). Abbreviations: DXA, dual-energy x-ray
762 absorptiometry; VL, vastus lateralis. Symbols: *, indicates increase from Pre to Post in older
763 participants ($p<0.05$); #, indicates different from younger, trained males ($p<0.05$).

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764 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

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

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770 Figure 1. mtDNA methylome data from older males prior to and following resistance training
771
772 Legend: Data represents differential methylation in older males following six weeks of resistance
773 training. Global mtDNA methylation significantly decreased following resistance training in
774 older males (panel A), which we show is unlikely due to any observed differences in read
775 coverage and association with CpG read density (panels B and C). Plotting methylation across
776 the ~16 kb mtDNA genome (panel D), we show clear differences in methylation between two
777 conditions (pre training, blue line; post training, red line). The four significant differentially
778 methylated regions are highlighted in grey bars, and a key regulatory region is indicated with an
779 asterisk. Data is N=10 for older males, and data for Figure 1D is presented as mean ± SEM.

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780 Figure 2. Methylation of the mtDNA regulatory region and correlational analyses with
781 phenotypic variables
782
783 Legend: Given that our analyses identified a differentially methylated region (DMR) spanning
784 the known regulatory region of the mtDNA, we further analyzed a 1.12 kb span within this
785 region (panel A). Here, we demonstrated that there was significant differential methylation (FDR
786 < 0.05) in older males with six weeks of training within this region (grey shaded region with 4/5
787 CpG sites with difference of >5%, panel B). The methylation of CpG sites residing within this
788 locus demonstrated significant correlations with phenotype data (indicated with asterisks) (panel
789 C). Dot coloration represents positive or negative associations, with size and strength of color
790 representing strength of the correlation coefficient. Comparison of mtDNA methylation of
791 younger, trained versus older males prior to training showed a clear hypomethylation ($P =$
792 0.022), whereas no such difference is observed with older males following training (panel D).
793 This is further represented across the mtDNA genome highlighting the restoration of the mtDNA
794 methylome to youth like levels via resistance training in older males (Figure 2E). Data is N=10
795 for all comparisons/correlations of older males, and N=7 for younger trained males. Data for
796 panels B and E are presented as mean ± SEM values.

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797 Figure 3. Mitochondrial transcript and marker adaptations with training in older males
798
799 Legend: These data represent mitochondrial transcript levels (panel A), TFAM mRNA levels
800 (panel B), TFAM protein levels (panel C), citrate synthase (CS) activity levels (panel E), and
801 protein levels of mitochondrial complexes I-V (panel F). Panels D and G contain representative
802 Western blots for data in panels C and F, respectively. qPCR data contain n=10 older males
803 prior to and following training, and n=7 younger trained males. Citrate synthase activity data
804 contain n=10 older males prior to and following training, and n=6 younger trained males.
805 Western blot contain n=9 older males prior to and following training, and n=6 younger trained
806 males. Symbols: *, indicates increase with training in older males ($p<0.05$); #, indicates different
807 from younger trained males prior to and following training ($p<0.05$). Gene abbreviations can be
808 found in-text. All data are presented as means ± SD values.

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809 Figure 4. Correlation between CpG methylation, gene expression and protein abundance in older
810 males prior to and following resistance training
811
812 Legend: Correlation of methylation in CpG sites residing within the 1.12 kb locus of interest in
813 older males prior to and following training shows significant (* = $P < 0.05$, ** $P<0.01$)
814 associations between methylation and gene expression (panel A, facet 1). Coloration of dots
815 represents direction of correlation, with strength of color and size of dot representing the strength
816 of correlation coefficients. Highlighted comparisons demonstrated a positive correlation between
817 CpG 1645 methylation and ND5 gene expression ($r = 0.57$, $P = 0.008$), and an inverse
818 correlation between CpG 162 methylation and 16S rRNA expression ($r = -0.59$, $P = 0.005$).
819 Correlation of methylation levels in older males prior to and following training with protein
820 abundance of complexes 3 and 4 yielded more striking associations (for all data with relative
821 significance see Suppl. File 4). Of note, CpG sites 163 to 16329 inversely correlated with
822 mtDNA complex 3 protein abundance ($P < 0.01$), with panel E highlighting the strongest
823 association within (CpG 16129; $r = -0.76$, $P = 0.0002$). Positive correlations between these data
824 sets also existed where, for example, CpG 106 methylation positively correlated with complex 4
825 (panel D; $r = 0.48$, $P = 0.044$). Data is N=10 for all comparisons/correlations.

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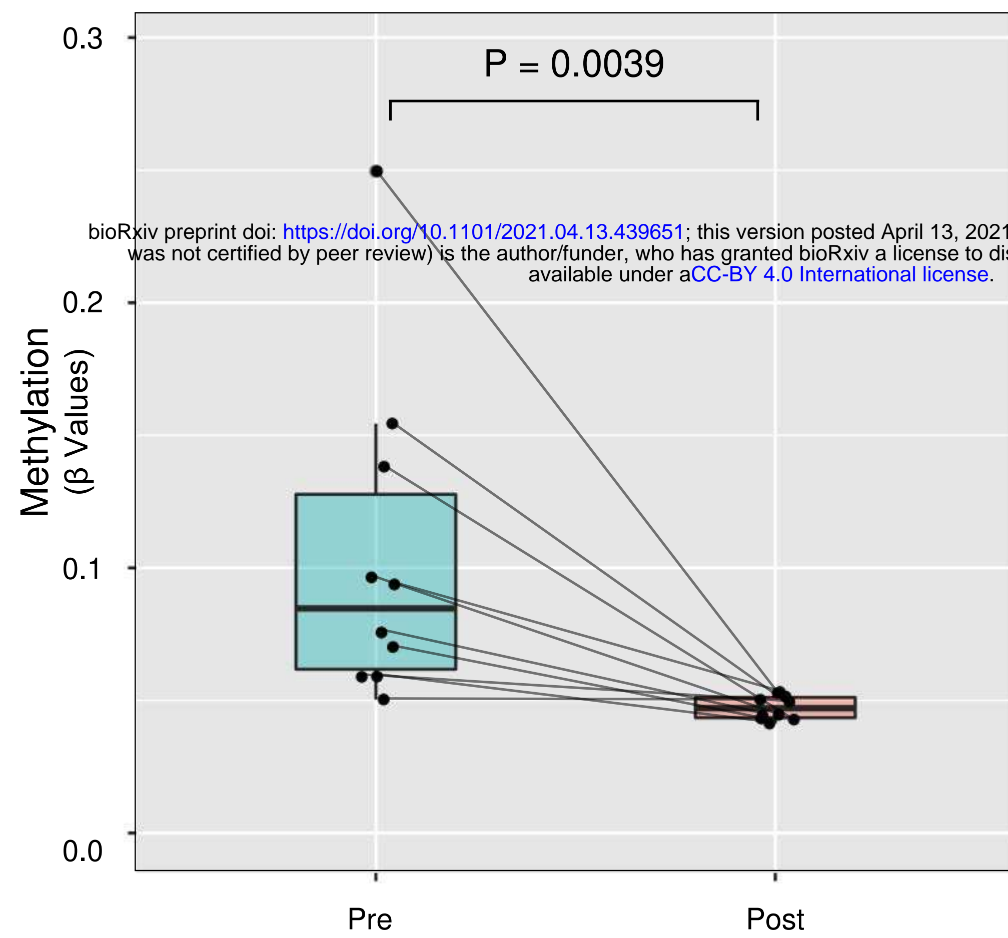

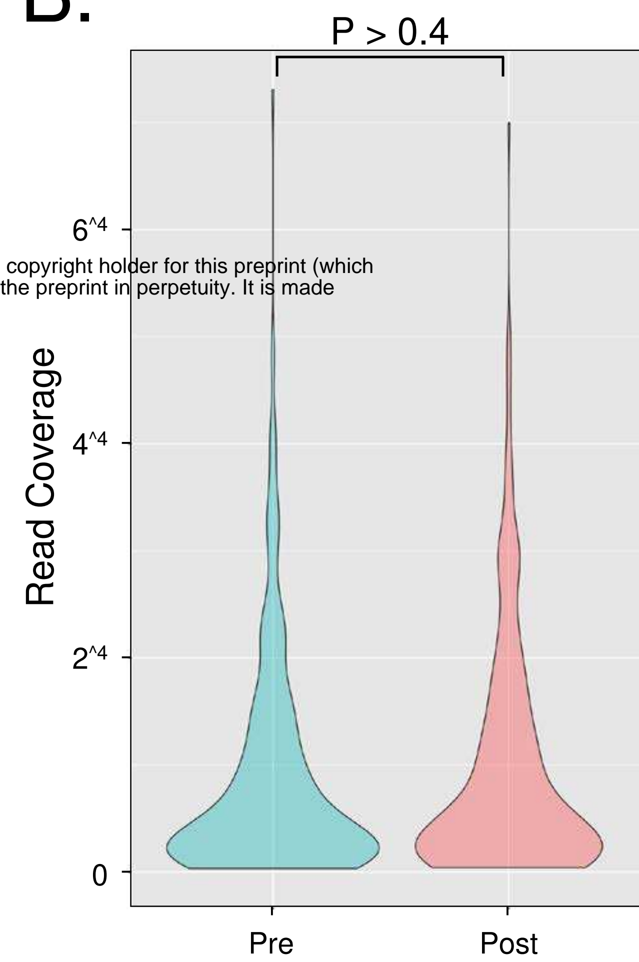

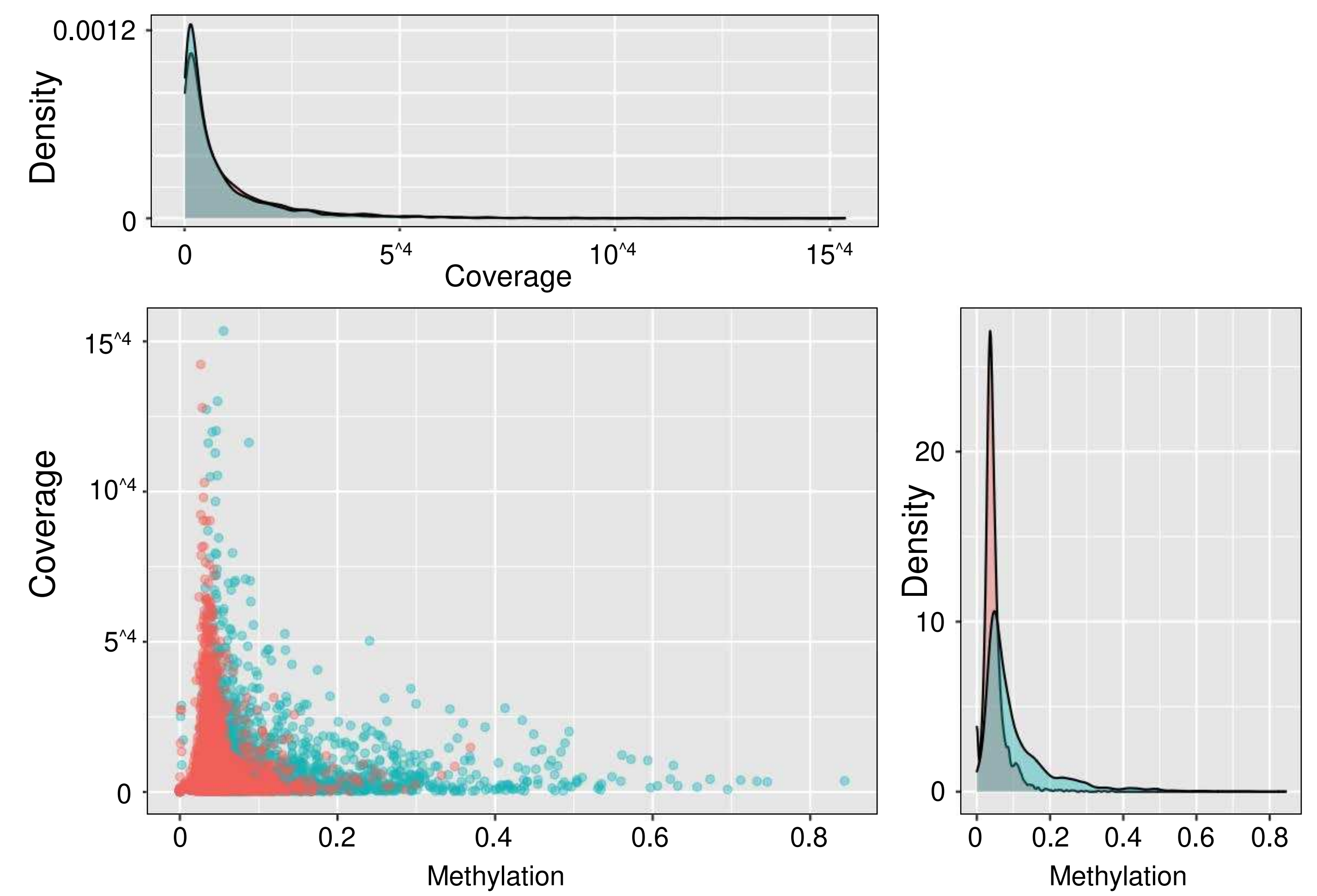

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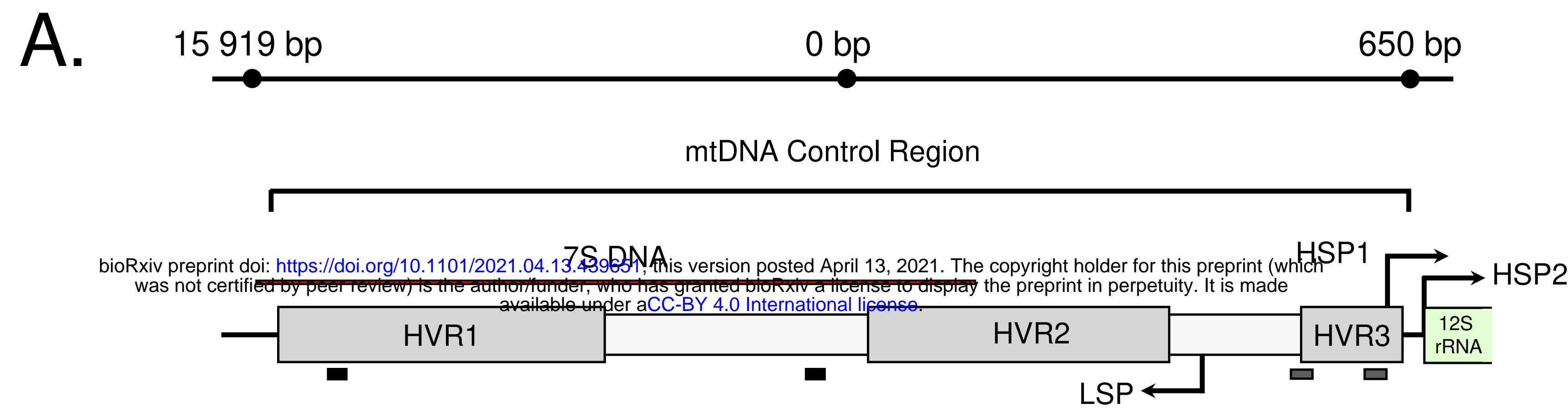
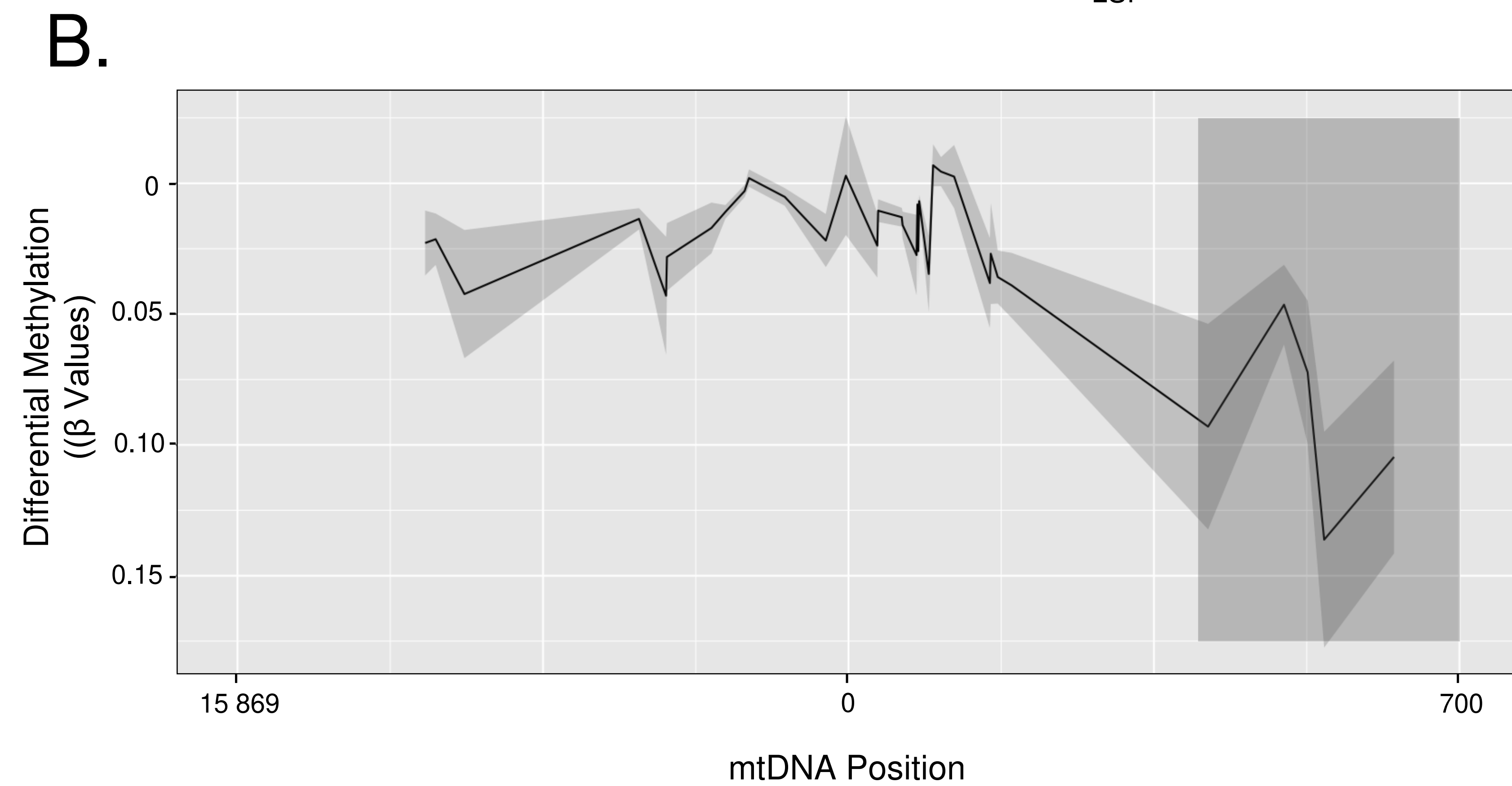
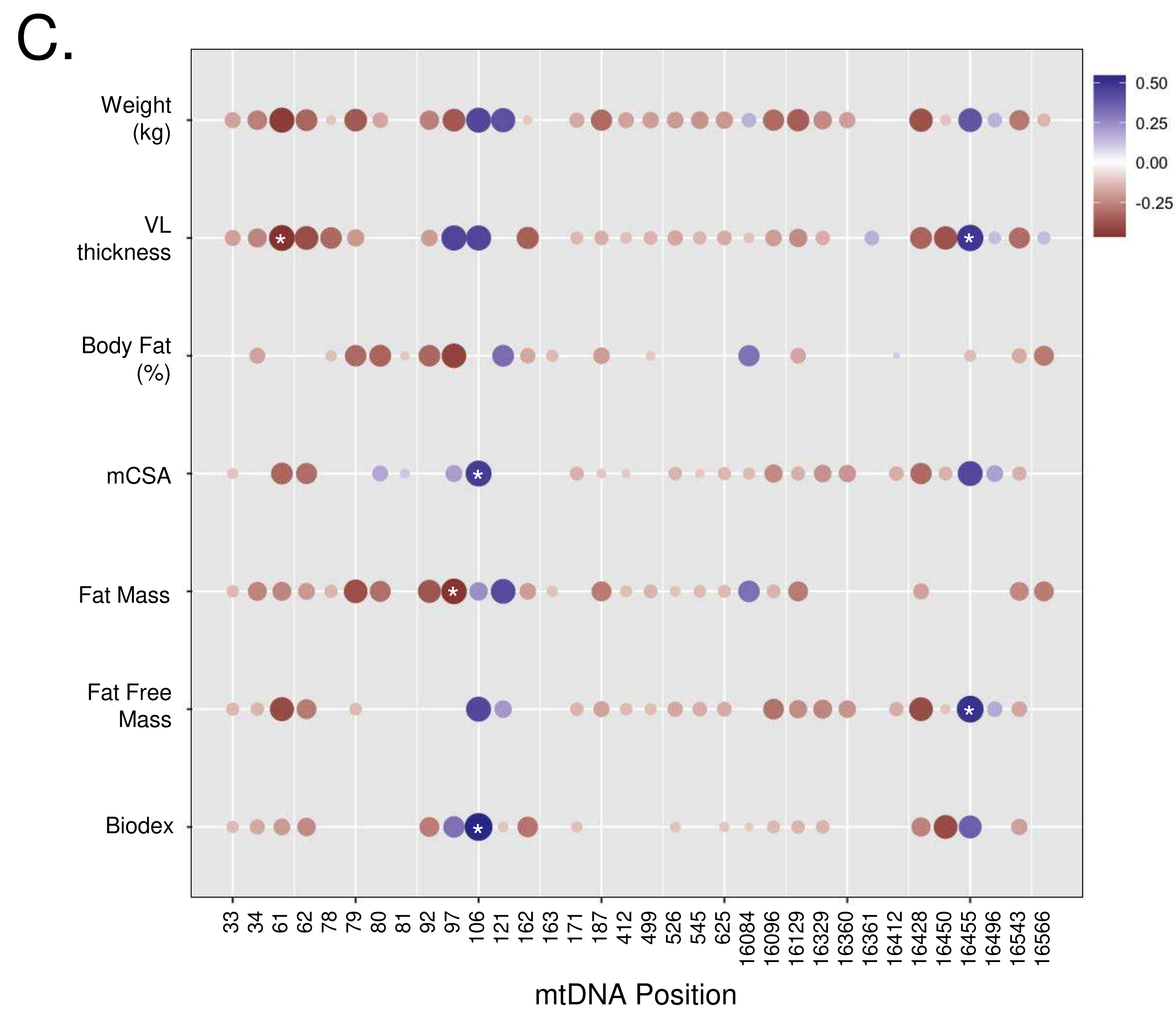
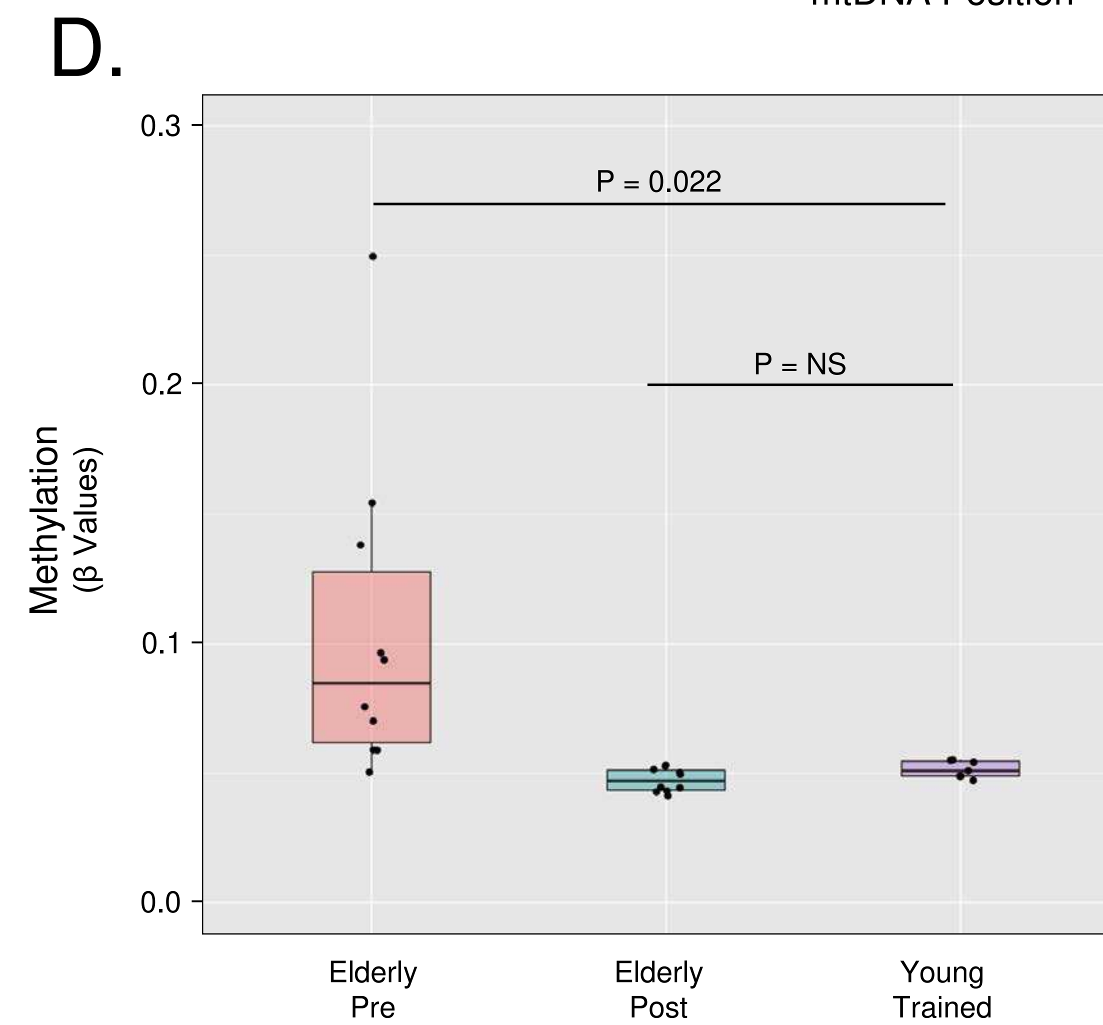
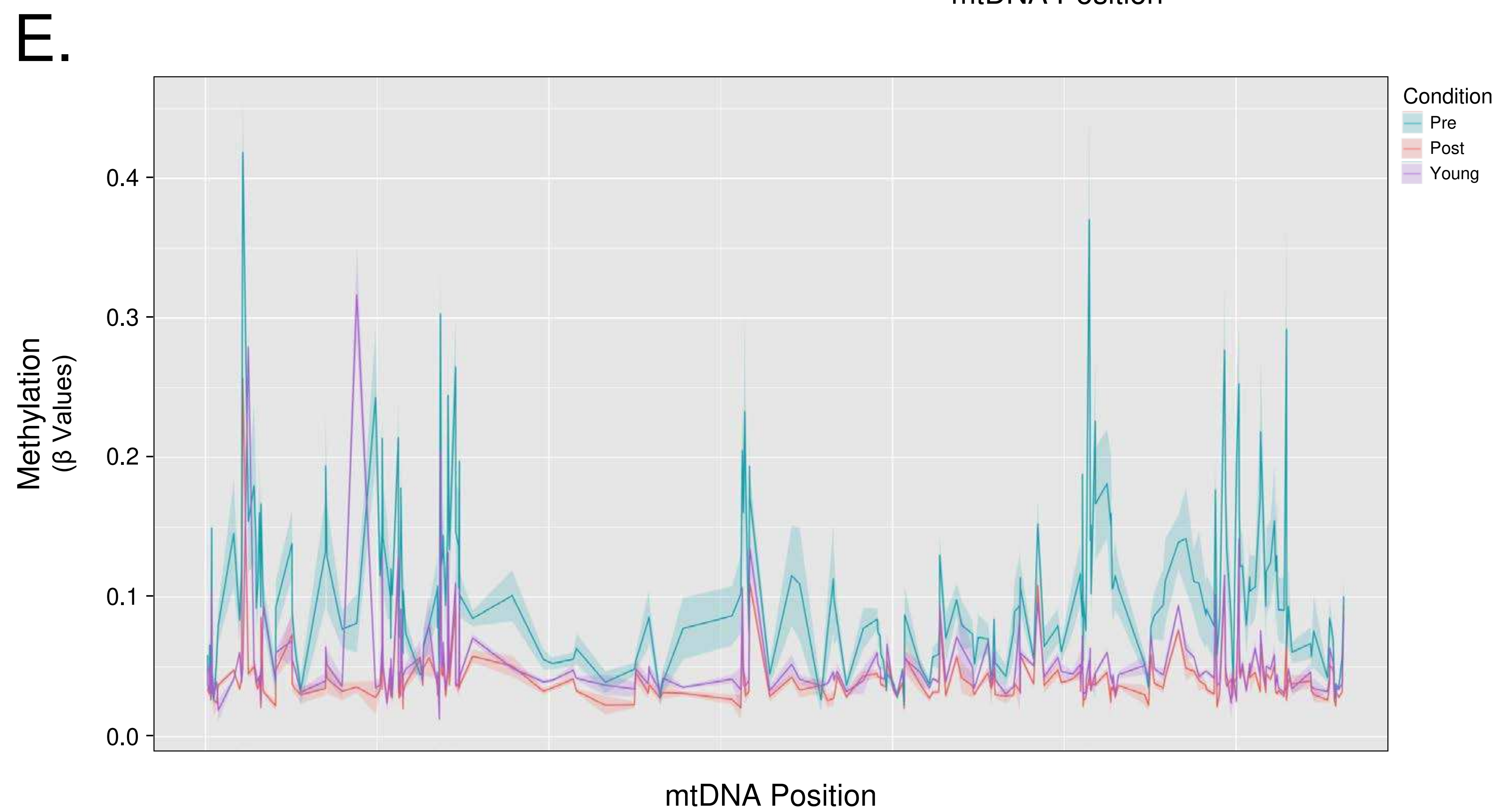
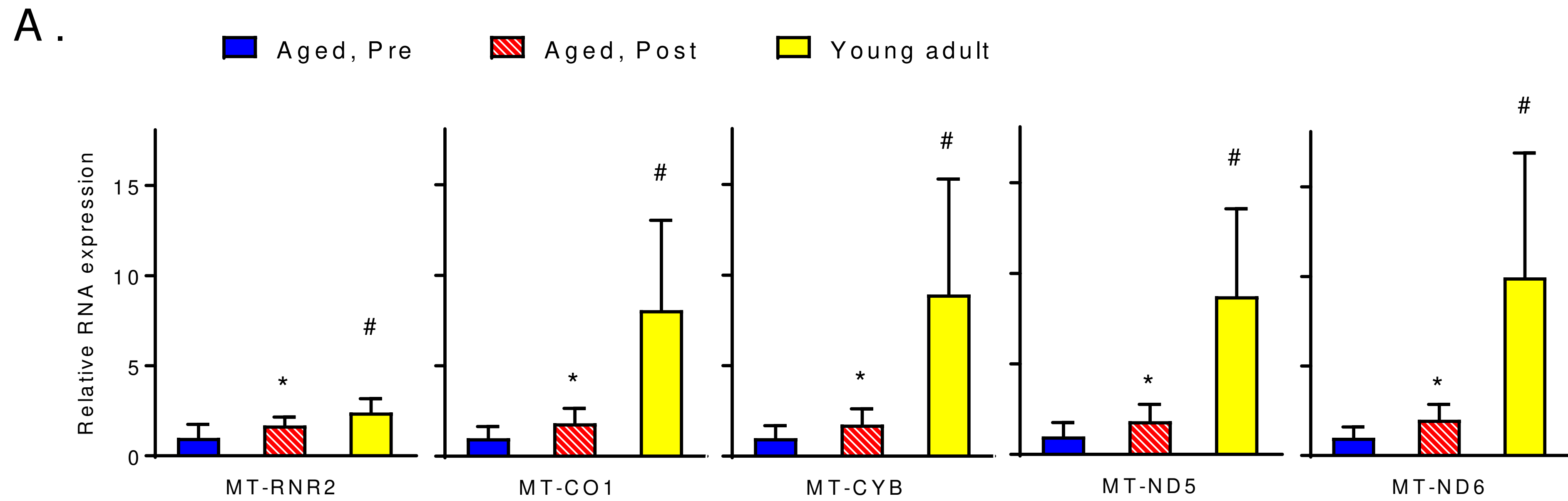


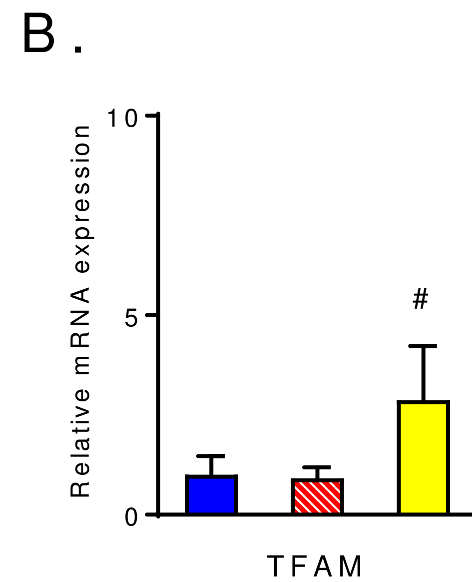


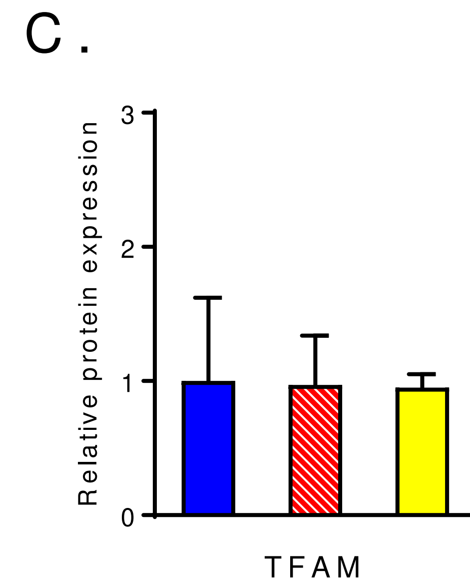


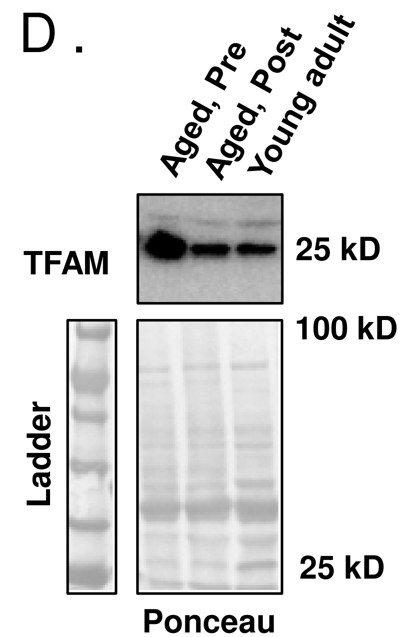


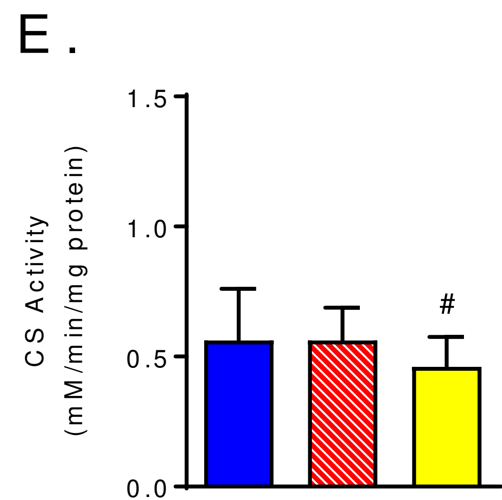


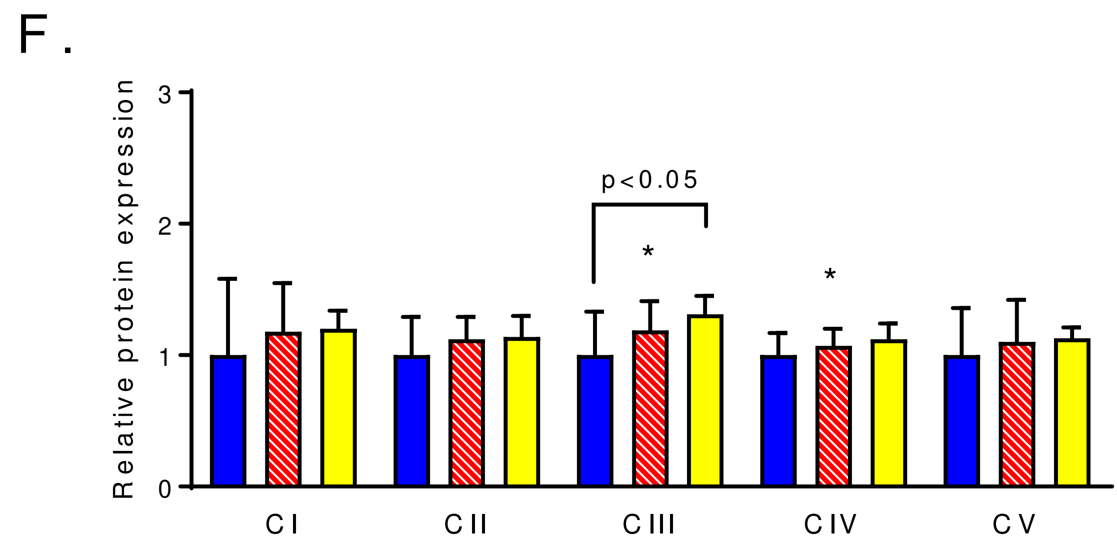

A.
Aged, Pre
Aged, Post
Young adult
Relative RNA expression
0
5
10
15
*
#
MT-RNR2
MT-CO1
MT-CYB
MT-ND5
MT-ND6

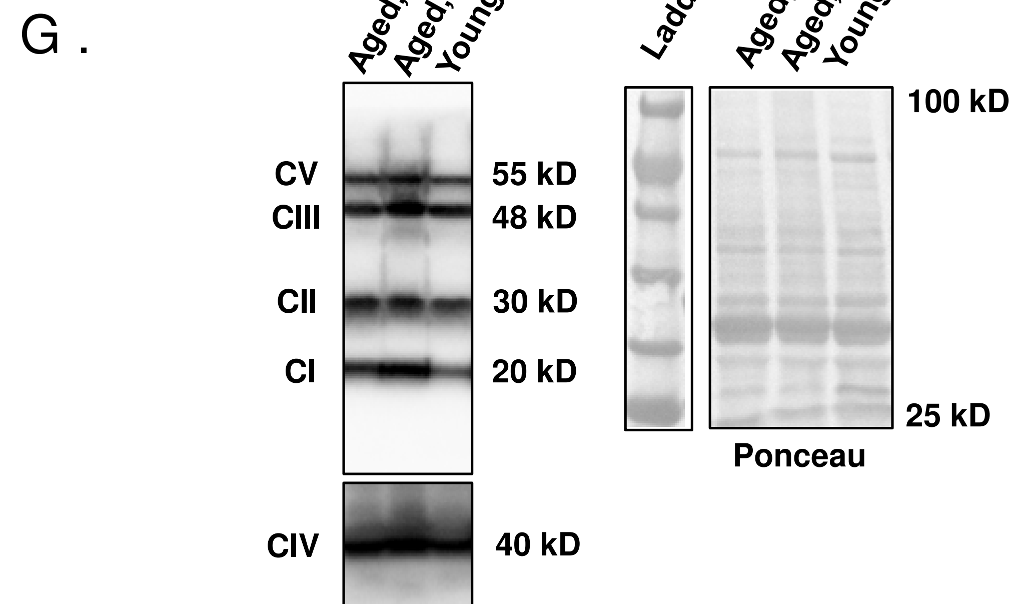

B.
Relative mRNA expression
0
5
10
#
TFAM


C.
Relative protein expression
0
1
2
3
TFAM


D.
Aged, Pre
Aged, Post
Young adult
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.
Aged, Pre
Aged, Post
Young adult
Ladder
CV
CIII
CII
CI
CIV
55 kD
48 kD
30 kD
20 kD
40 kD
100 kD
25 kD
Ponceau

A.

ND6
ND5
CYTB
COX1
16S rRNA
mito. Complex 4 protein
mito. Complex 3 protein
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

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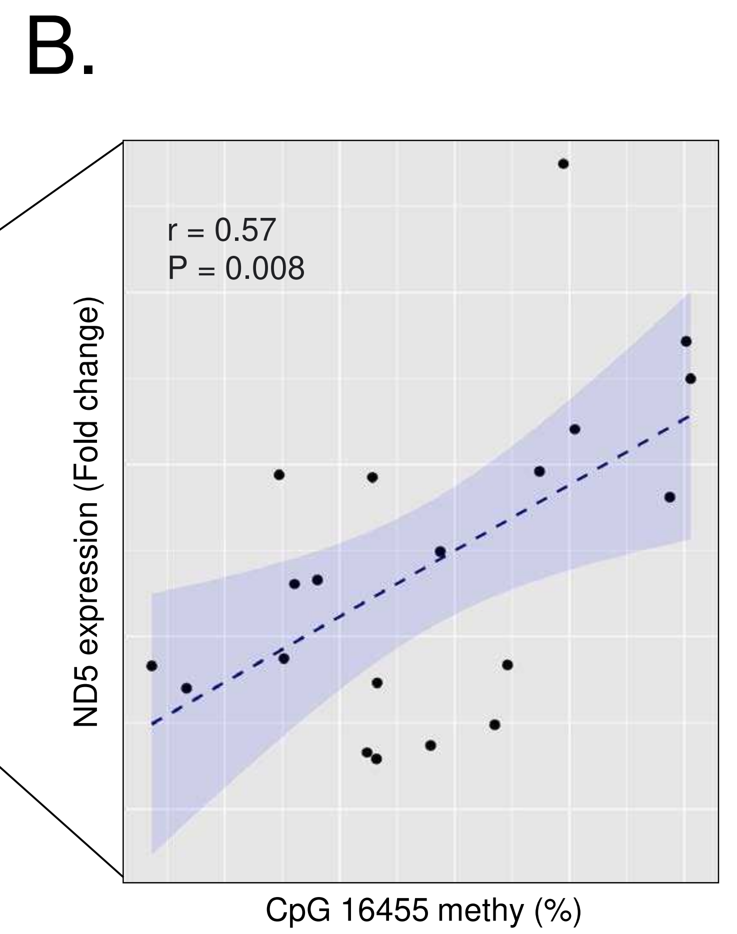
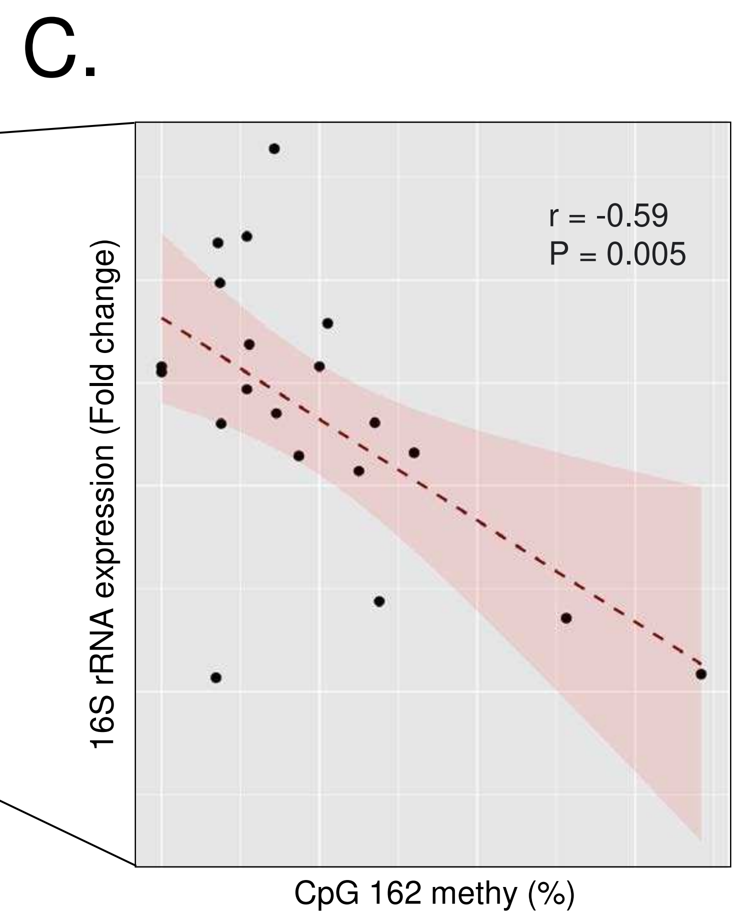
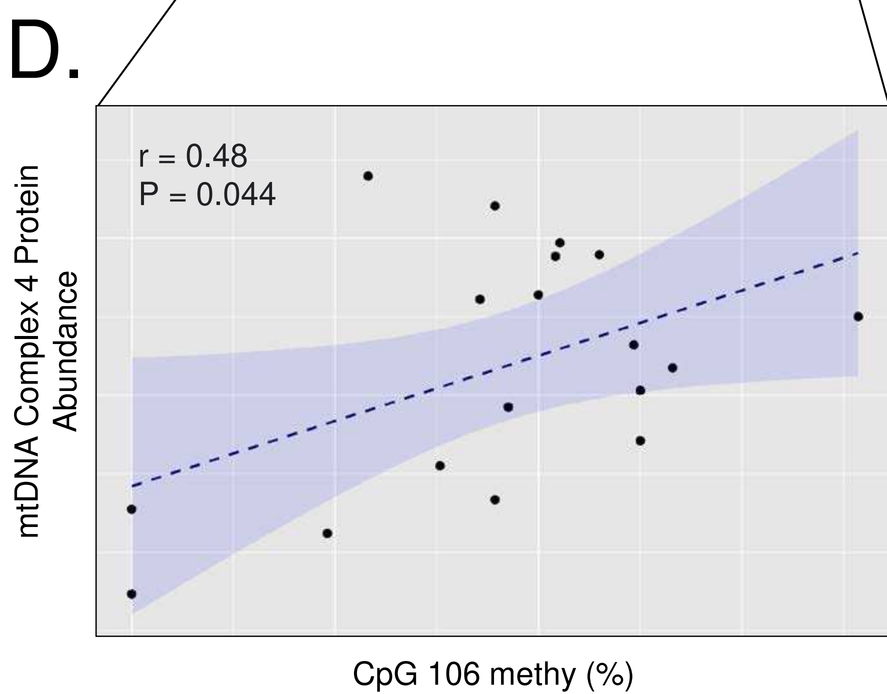
B.



C.



D.



E.