

1 **Effects of fasting induced carbohydrate depletion on murine ischemic skeletal**
2 **muscle function.**

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23 **Abstract**

24 Stored muscle carbohydrate supply and energetic efficiency constrain muscle functional
25 capacity during exercise and are influenced by common physiological variables (e.g. age,
26 diet, and physical activity level). Whether these constraints affect overall functional
27 capacity or the timing of muscle energetic failure during acute ischemia is not known. We
28 interrogated skeletal muscle contractile properties in two anatomically distinct hindlimb
29 muscles that have well characterized differences in energetic efficiency (locomotory-
30 extensor digitorum longus (EDL) and postural- soleus muscles) under conditions of
31 reduced carbohydrate supply. 180 mins of acute ischemia resulted in complete energetic
32 failure in all muscles tested, indicated by: loss of force production, substantial reductions
33 in total adenosine nucleotide pool intermediates, and increased adenosine nucleotide
34 degradation product - inosine monophosphate (IMP). These changes occurred in the
35 absence of apparent myofiber structural damage assessed histologically by both
36 transverse section and whole mount. Restriction of the available intracellular carbohydrate
37 pool by fasting (~50% decrease in skeletal muscle) did not significantly alter the timing to
38 muscle functional impairment or affect the overall force/work capacities of either muscle
39 type. Fasting did cause rapid development of passive tension in both muscle types, which
40 may have implications for optimal timing of reperfusion or administration of precision
41 therapeutics.

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49 **Introduction**

50 Ischemic skeletal muscle necrosis occurs concurrently with several common clinical
51 conditions (e.g. peripheral arterial disease, compartment syndrome, or diabetic necrosis)
52 and is a complicating factor of successful muscle graft transplantation(1–3). The severity
53 of necrosis during an ischemic episode has long been considered a sole function of time,
54 temperature, and magnitude of the hypoxic insult(4,5). However, the timing of the events
55 that precede irreversible functional impairment and necrosis during ischemia may also
56 depend on other key variables including: metabolic rate; contractile efficiency; and the size
57 of the stored carbohydrate pool(4). Carbohydrate metabolism is key, as muscle energy
58 supply becomes dependent on anaerobic fermentation of stored carbohydrate sources
59 during ischemia(6–8). Glycogen is the primary storage form of carbohydrate in skeletal
60 muscle, and its storage/utilization can be influenced by acute environmental factors as
61 well as chronic diseases(9–14).

62

63 Previous studies have examined the time dependent changes of metabolites and
64 contractile function in rodent skeletal muscle following ischemia with reperfusion (I/R)(15–
65 18). Several important observations can be gleaned from these studies: First, locomotory
66 (fast glycolytic) muscles experienced more damage compared to postural (slow oxidative)
67 muscles(15,17). Second, The degree of initial injury can have large effects on post
68 ischemic recovery time(16). Lastly, Optimal reperfusion timing is related to changes in
69 muscle metabolite levels during ischemia(18). A major limitation of I/R studies is that it is
70 difficult to distinguish between the functional impairment and/or damage that is attributable
71 to the ischemia itself versus that caused by the reperfusion injury.

72

73 In a previous study, using an *in vivo* mouse hindlimb ischemia model (without reperfusion),
74 we found that myonecrosis develops between three and six hours after the onset of

75 ischemia and is accompanied by a complete loss of contractile function(19). This led us
76 to examine the <3-hour time domain in this study to better characterize the exact temporal
77 nature of muscle functional impairments and metabolite changes that occur under
78 ischemic conditions. We hypothesized that reductions in stored muscle glycogen would
79 significantly shorten the amount of time that the muscles could remain functional during
80 ischemia. To test this hypothesis, we utilized fasting to induce an approximate 50%
81 decrease in resting muscle glycogen and employed a carefully controlled experimental
82 system to assess the effects of carbohydrate depletion on isolated mouse hindlimb muscle
83 function during severe hypoxia and nutrient deprivation. Our data provide a novel
84 characterization of ischemic muscle mechanical/energetic failure and paint a detailed
85 picture of the timing of these impairments. This information will provide a valuable
86 resource to be used in conjunction with studies of ischemia/reperfusion in mouse hindlimb
87 ischemia models.

88

89 **Materials and Methods**

90

91 **Animals**

92 Adult male BALB/c mice (N=32), aged 16-24 weeks old, were obtained from Jackson
93 Laboratories (Bar Harbor, ME). All work was approved by the Institutional Animal Care
94 and Use Committee of East Carolina University. Animal care followed the Guide for the
95 Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources,
96 Commission on Life Sciences, National Research Council. Washington: National
97 Academy Press, 1996. Animals had free access to water and food except during fasting
98 protocols, during which animals had free access to water only.

99

100 **Laser scanning confocal and multiphoton microscopy**

101 For microvascular imaging, Dylight 594 conjugated *Griffonia simplicifolia* isolectin B₄ (GS-
102 IB₄) (Vector Labs, Burlingame, CA) was injected retro-orbitally one hour prior to sacrifice.
103 EDL and soleus muscles were dissected, and immersion fixed in 4% paraformaldehyde.
104 BODIPY and DAPI staining was performed as previously described(20). Sarcomeric actin
105 staining was performed in PFA fixed whole mount muscles, following permabilization with
106 30µg/ml saponin, using 200nM Alexa Fluor 488 conjugated phalloidin (Thermo Fisher,
107 Waltham MA). For NAD(P)H autofluorescence imaging, live muscle was dissected
108 following sacrifice and mounted at resting length. Live muscles were imaged in a glass
109 bottom dish in Krebs Ringer solution. All imaging was performed using an Olympus
110 FV1000 laser scanning confocal microscope (LSCM). Acquisition software was Olympus
111 FluoView FSW (V4.2). The objective used was 60X oil immersion (NA=1.35, Olympus
112 Plan Apochromat UPLSAPO60X(F)) or 30X (NA= 1.05, Olympus Plan Apochromat
113 UPLSAPO30XS). Images were 800x800 pixel with 2µs/pixel dwell time. Detector noise
114 was reduced by application of a 3X line scanning kalman filter. Images were acquired in
115 sequential scan mode. 2µM DAPI was used for nuclear counterstaining (Sigma Aldrich,
116 St. Louis, MO) and was excited using the 405nm line of a multiline argon laser; emission
117 was filtered using a 490nm dichroic mirror and 430-470nm barrier filter. BODIPY and
118 AF488-phalloidin were excited using the 488nm line of a multiline argon laser; emission
119 was filtered using a 560nm dichroic mirror and 505-540nm barrier filter. Dylight 594 (GS-
120 IB₄) was excited using a 559nm laser diode; emission was filtered using a 575-675nm
121 barrier filter. Zero detector offset was used for all images. The pinhole aperture diameter
122 was set to 105µm (1 Airy disc). NAD(P)H autofluorescence has been shown to be highly
123 localized to skeletal muscle mitochondria(21). NAD(P)H autofluorescence was excited
124 using a mode locked pulsed laser (Mai Tai, Spectra Physics, Santa Clara, CA) tuned to
125 720nm. Emission was collected using separate non-descanned detectors.

126

127 **Dystrophin/Laminin immunofluorescence in transverse muscle sections**

128 EDL and soleus muscles were embedded in optimal cutting temperature medium (OCT),
129 and frozen in liquid nitrogen cooled isopentane for cryosectioning. 10µm sections were
130 cut using a CM-3050S cryostat (Leica, Wetzlar Germany) and collected on charged glass
131 slides. Sections were then fixed in 1:1 acetone/methanol for 10 minutes at -20°C,
132 rehydrated in 1X phosphate buffered saline (PBS), and blocked in 5% goat serum + 1X
133 PBS for one hour at room temperature. Sections were then incubated with mouse anti-
134 human monoclonal dystrophin antibody (Thermo-Fisher, MA5-13526), and rabbit anti-rat
135 primary laminin antibody (Thermo-Fisher, A5-16287) at 4°C overnight. Sections were
136 washed 3X for 10 minutes with cold 1X PBS and incubated for 1 hour with Alexa-fluor 594
137 conjugated goat anti-rabbit IgG or Alexa-fluor 488 conjugated goat anti-mouse (highly
138 cross adsorbed) IgG2b secondary antibody (1:250, Invitrogen). Sections were mounted
139 using Vectashield hard mount medium without Dapi (Vector Labs). Images were taken
140 with an Evos FL auto microscope (Thermo Fisher, Waltham, MA) with a plan fluorite 20X
141 cover slip corrected objective lens (NA = 0.5, air). The following excitation/emission filter
142 cubes were used: GFP (470/22 nm Excitation; 510/42 nm Emission) and Texas Red
143 (585/29 nm Excitation; 624/40 nm Emission). 4X and 20X magnification images were
144 taken for each condition. Image processing was performed using ImageJ (NIH,
145 v1.51f)(22).

146

147 **Fasting**

148 Pilot testing was performed to determine the minimal fasting time to achieve an ~50%
149 reduction in resting skeletal muscle glycogen(23). Muscle glycogen reached the target
150 reduction after 24 hours of fasting (one dark cycle).

151

152 **Measurement of muscle mechanical function**

153 Mice were sacrificed by cervical dislocation under isoflurane anesthesia (confirmed by
154 lack of pedal withdrawal reflex). Extensor digitorum longus (EDL) or soleus muscles were
155 carefully dissected and tied at both tendon ends with 5-0 silk sutures (Thermo Fisher,
156 Waltham, MA). Muscles were tied to an anchor at the proximal end and a dual mode force
157 transducer (Aurora 300B-LR, Aurora, ON, Canada) at the distal end in a vertical bath at
158 22°C. All protocols were performed in the absence of additional carbon fuel sources (i.e.
159 amino acids, glucose, etc.) to restrict muscles to stored fuel supplies. The bath solution
160 was a modified Krebs Ringer solution described previously(24). All muscles were
161 dissected and mounted within 15 mins of sacrifice. Muscles were equilibrated in the bath
162 for 10 mins, and optimal length (L_0) was determined by stimulating twitch contractions
163 (0.2ms pulse width, 1 pulse/train) at 10 second intervals and adjusting the length
164 incrementally until maximal force was achieved. Supramaximal stimulation voltage for
165 both muscle types was determined to be 20V. L_0 (mm) was measured using a digital
166 microcaliper (Thermo Fisher, Waltham, MA). A force frequency curve was developed for
167 each muscle using stepwise increasing stimulation frequencies of 10, 20, 40, 60, 80, 100,
168 and 120 Hz (.2ms pulse width, pulses/train=half of the stim. Freq.). Baths were aerated
169 with 95% O_2 /5% CO_2 (oxygenated; O_2 condition) during L_0 determination and the initial
170 force frequency curve. The aeration source was then either left the same or changed to
171 95% N_2 /5% CO_2 (hypoxic; N_2 condition) to simulate ischemia. The muscles were then
172 equilibrated for 10 mins, and an initial isokinetic contraction protocol was elicited in the O_2
173 condition (100Hz isometric contraction for 0.8 seconds followed by a 3mm shortening
174 phase over .3 seconds, then a return to L_0 over 30s for the EDL; for the soleus 80Hz
175 isometric contraction was elicited for 0.8 seconds followed by a 4mm shortening phase
176 over .4 seconds, then a return to L_0 over 30s). The aeration source was then either left the
177 same or changed to 95% N_2 /5% CO_2 (hypoxic condition), with experimental conditions
178 alternated each time to reduce bias. The muscles were then equilibrated for 10 mins,

179 followed by stimulated isokinetic contractions every 10 mins for 180 mins (18 total
180 contractions). We chose this timing based on our previous observation that excitation
181 contraction coupling is impaired in muscles isolated from BALB/c mice 180 minutes after
182 induction of acute hindlimb ischemia (in the absence of histological signs of tissue
183 necrosis)(19). A second force frequency curve was measured following the 180-min.
184 isokinetic protocol without changing the aeration source. Muscles were removed from the
185 apparatus, blot dried on paper, weighed, and flash frozen in liquid nitrogen for biochemical
186 analyses. Isometric time-tension integrals (TTI) were calculated by integrating over the
187 isometric (phase I) portion of the curve and are expressed in units of N*s/cm². Isokinetic
188 work (W) was obtained by integrating the force over the length change during the
189 shortening (phase II) portion of the protocol and is expressed in units of J/cm².

190

191 Absolute isometric force measurements were normalized to mathematically approximated
192 cross-sectional areas of the muscles. The cross-sectional area for each muscle was
193 determined by dividing the mass of the muscle (g) by the product of its optimal fiber length
194 (L_f , cm) and estimated muscle density (1.06 g cm⁻³). Muscle force production was
195 expressed as specific force (N/cm²) determined by dividing the tension (N) by the
196 calculated muscle cross-sectional area. L_f was obtained by multiplying L_0 by the standard
197 muscle length to fiber length ratio (0.45 for adult mouse EDL; 0.71 for soleus)(25). A gas
198 calibrated Clark electrode (Innovative instruments, Lake Park, NC) was used to assess
199 the oxygen saturation of the isolated bath medium under both aeration conditions prior to
200 carrying out the experiments. O₂ conditions were approximately 90% saturation measured
201 at the center of the bath (after 10 mins of aeration). N₂ conditions were <2% saturation.

202

203 **Measurement of glycogen content in whole tissue**

204 Skeletal muscle and liver tissues were flash frozen in liquid nitrogen and stored at -80°C.
205 Glycogen assays were performed using acid hydrolysis and an enzyme coupled
206 assay(26). Briefly, tissue samples were digested/hydrolyzed under acidic conditions using
207 2N hydrochloric acid (Sigma Aldrich, St. Louis, MO) on a heating block at 95°C for 2 hours
208 with additional vortexing. Samples were neutralized with equal volume 2N sodium
209 hydroxide (Sigma). A small amount of tris HCl pH 7.0 (~1% of final volume) was added to
210 buffer the solution. Samples were added to a clear 96 well plate in duplicate and were
211 incubated with a solution containing: >2000U/L hexokinase (*S. cerevisiae*), >4000 U/L
212 NAD⁺ dependent glucose-6-phosphate dehydrogenase (*L. mesenteroides*), 4mM ATP,
213 2mM Mg²⁺, and 2mM NAD⁺ (Hexokinase reagent solution; Thermo Fisher). Water was
214 used in place of the reagent for background correction. A standard curve of D-glucose
215 (Sigma Aldrich) was used to calculate the concentrations of hydrolyzed glucosyl units in
216 each sample. Colorimetric measurement of NAD(P)H absorbance was made at 340nm
217 using a Cytation 5 microtiter plate reader (Biotek, Winooski, VT). Liver samples were
218 diluted 1:50 in water prior to enzyme coupled assays to obtain absorbance values within
219 the range of the standard curve. Data were normalized to tissue mass and represented
220 as nmoles glucose/mg tissue wet weight. The response coefficient (R_{Glyc}) is defined as the
221 fractional change in experimental group mean relative to the basal group (i.e. Mean Basal
222 – Mean Experimental/Mean Basal*100).

223

224 **Ultra-performance liquid chromatography (UPLC) measurements of adenosine** 225 **nucleotides in whole tissue**

226 UPLC measurements of adenosine nucleotides in whole muscle tissue have been
227 described in detail previously(27). Briefly, isolated muscles were flash frozen in liquid
228 nitrogen, homogenized in ice-cold perchloric acid using a glass on glass homogenizer,
229 and centrifuged to remove precipitated proteins. Samples were neutralized using

230 potassium hydroxide and centrifuged a second time, to remove perchlorate salt.
231 Adenosine nucleotides and degradation products were assayed using an Acquity UPLC
232 H class system (Waters, Milford, MA). Metabolites were identified by comparison of peak
233 retention times of pure, commercially available standards (Sigma–Aldrich). These UPLC
234 measures can provide an index of intracellular energetic state. The amount of IMP reflects
235 longer periods of metabolic demand exceeding supply as the available adenylate pool is
236 decreased via irreversible deamination of AMP to IMP. Over the timeframe of these
237 stimulation protocols, IMP accumulation is a reliable measure of sustained mismatch
238 between ATP supply and demand. (Adenosine triphosphate-ATP, adenosine
239 diphosphate-ADP, adenosine monophosphate-AMP, and inosine monophosphate-IMP).
240

241 **Statistical Analysis**

242 Results of statistical comparisons are represented by means \pm standard error (SEM).
243 Sample variance (in figure panels) is represented by sample standard deviation (SD).
244 Analyses and plotting were carried out using Graphpad prism (V8.01; Windows 10).
245 Unpaired two-tailed t-tests were used for between group comparisons. For comparison of
246 means, p values of < 0.05 were considered statistically significant.

247

248 **Results**

249 Extensor digitorum longus (EDL) and soleus muscles were chosen for their known
250 differences in thermodynamic efficiency(28). The muscles also characteristically rely on
251 different modes of energy metabolism (glycolytic and oxidative metabolism
252 respectively)(29). The specialized nature of each muscle is highlighted for illustrative
253 purposes by whole mount imaging (**Fig 1**), contrasting the dramatically different
254 microvascular anatomy, cellular lipid droplet distribution (BODIPY), and mitochondrial
255 density/distribution (NAD(P)H).

256 **Fig 1: Microanatomy of Extensor digitorum longus (EDL) and soleus muscles differs**
257 **in several key ways.** Qualitative images highlighting a few of the key anatomical
258 differences between the fast twitch extensor digitorum longus (EDL) and slow twitch
259 soleus muscles (A). From left to right: (Left) Vessel density images are z-projections of
260 Dylight 594 conjugated lectin; (Middle) BODIPY images are z-projections of BODIPY
261 positive lipid droplets. Red signal in BODIPY images are lectin stained blood vessels. Blue
262 signal in BODIPY images are myonuclei. Arrows indicate BODIPY positive lipid droplets
263 in zoomed image inlays; (Right) Mitochondrial NAD(P)H images are optical sections of
264 reduced pyridine nucleotide autofluorescence in live isolated skeletal muscle. NAD(P)H
265 fluorescence intensity is mapped onto the image (highest intensity in white). Scale bars
266 are 25um.

267

268 Fasting is a well characterized and effective method of whole body carbohydrate depletion
269 in mice, due to their high thermal conductivity and large surface area to body volume
270 ratio(23). This method was chosen for this study because it is independent of the
271 confounding effects of exercise or contraction induced fatigue(30). The mean change in
272 bodyweight over the fasted period (24 hours) was 3.9 ± 0.12 grams, approximately 13%
273 of the mean initial weight. We observed a large difference in stored glycogen levels
274 between fed and fasted groups in both liver (~90% lower) (**Table 1**) and skeletal muscle
275 (~50% lower) (**Table 1**). Interestingly, the resting glycogen concentration was higher in
276 the soleus than the EDL under both fed and fasted conditions. Additionally, soleus
277 muscles had a lower mean glycogen concentration in the fasted group relative to the fed
278 state (mean percent difference of 41.6% compared to 56.1% in the EDL groups; **Table 1**).

279

280

281 **Table 1: Basal tissue glycogen concentrations in the liver and skeletal muscle of fed Vs. fasted**
282 **groups.**

Tissue	Condition	Glycogen (nmol/mg)	StDev (nmol/mg)	% Fed Group
Liver	Fed	387.9*	88.43	
	Fasted	42.0*	20.7	10.8
EDL	Fed	34.4*#	8.0	
	Fasted	19.3*#	3.2	56.1
Soleus	Fed	61.9*#	17.5	
	Fasted	25.8*#	3.6	41.6

283 Units are nanomoles hydrolyzed glucosyl units/milligram tissue wet weight (nmol/mg). *p<.05 Fed
284 V. Fasted Groups. #p<.05 EDL V. Sol. N=4. Sample standard deviation (StDev).

285

286 Fasting had no effect on the isometric force-frequency relationship at baseline or under
287 any of the tested conditions in the EDL (**Fig 2A**) or soleus (**Fig 2B**), indicating reduced
288 carbohydrate pool size did not alter excitation-contraction coupling. Specific force values
289 for both muscles were consistent with those obtained previously(24). Additionally, we
290 observed characteristic reductions in maximal specific force following the O₂ protocols
291 (and completely impaired force production following the N₂ protocols) in both muscles (**Fig**
292 **2A,B**). Notably, the isometric force capacity during each protocol did not differ between
293 the fed and fasted groups in either the EDL (**Fig 2C**) or the soleus (**Fig 2D**). Similarly, the
294 work capacity over the course of the protocols did not differ for either muscle between the
295 fed and fasted states (**Fig 2E,F**). As expected, the force and work capacities were greatly
296 reduced under the N₂ conditions compared to O₂ conditions.

297

298 **Fig 2: Effects of carbohydrate depletion on excitation-contraction coupling and**
299 **force/work capacities.** Specific force-frequency curves for EDL (A) and soleus (B). Basal
300 conditions are 95% O₂ prior to isokinetic protocol. (C,D) Specific force capacities were
301 obtained by summing the isometric portion of the time-tension integrals at each sampling
302 interval for the EDL and soleus respectively. (E,F) Specific work capacities were obtained
303 by summing the isovelocity (shortening) portion of the length-tension integrals at each
304 sampling interval for the EDL and soleus. N=8/treatment/group. Data are presented as
305 mean ± SD. *p<.05 O₂ Vs. N₂ (Vs. Basal A,B).

306

307

308 Given that no substantial differences in force or work capacities were observed, we next
309 examined whether the timing of muscle functional impairments would differ between the
310 fed and fasted states. The time-tension integral (TTI) of the isometric portion of each
311 contraction was plotted as a function of the number of contractions (or time) during each
312 protocol for the EDL (**Fig 3A**) and soleus (**Fig 3B**). This measurement represents the
313 ability of the muscle to perform sustained non-shortening contractions. Additionally, the
314 length-time integral of the isokinetic portion of each contraction was also plotted against
315 the number of contractions for the EDL (**Fig 3C**) and soleus (**Fig 3D**). This measurement
316 represents the ability of the muscle to perform shortening work. Both sets of curves were
317 characterized by an inverse linear relationship under O₂ conditions and a distinctly non-
318 linear inverse relationship under N₂ conditions during the time and frequency domains of
319 the experiments. The muscles from the fasted group experienced more rapid reduction in
320 both TTI and work. All the tested muscles, however, experienced full impairment (defined
321 as force or work output <10% of the initial value) within a relatively small (~10-20 min.)
322 window of time. Passive tension was measured at the start of each contraction for the EDL
323 (**Fig 3E**) and soleus (**Fig 3F**). This measurement represents stiffening of the muscle, which

324 may be due to several possible factors, including impaired calcium reuptake or cellular
325 swelling due to uncontrolled fluid uptake(31). None of the muscles experienced substantial
326 changes in passive tension during the O₂ protocol. Large increases in passive tension
327 occurred in both muscles under N₂ conditions. Interestingly, passive tension development
328 occurred earlier in the fasted groups (**Fig 3E,F**). To account for the possibility that the
329 muscles were accumulating excessive water, the wet weights of the EDL (**Fig 3G**) and
330 soleus (**Fig 3H**) were plotted. No differences in wet weight between the fed and fasted
331 states were observed in either muscle, and all the tested muscles accumulated additional
332 weight following the N₂ protocol.

333

334 **Fig 3: Effects of carbohydrate depletion on the timing of functional impairment**
335 **during ischemia.** Isometric time-tension integrals (TTI) of each contraction over the
336 course of 18 contractions (or 180 minutes) under each condition for the EDL (A) and
337 soleus (B). Isokinetic length-tension integrals (isokinetic work) of each contraction for the
338 EDL (C) and soleus (D). Developed passive tension (measured at the start of each
339 contraction) for the EDL (E) and soleus (F). Muscle wet weights obtained at the end of
340 each protocol for the EDL (G) and soleus (H). N=8/treatment/group. Data are presented
341 as mean ± SD.

342

343 We next measured the muscle glycogen levels following the O₂ and N₂ protocols. The N₂
344 protocol reduced glycogen concentrations in all the muscles tested, relative to the O₂
345 condition (**Table 2**). Additionally, glycogen concentrations were lower in the fasted soleus
346 groups compared to the fed groups under both O₂ and N₂ conditions (**Table 2**). However,
347 glycogen concentrations did not differ between fed and fasted groups in the EDL muscles.
348 Using the response coefficient (R_{Glyc}), allowed for comparison of each group mean to the

349 basal values that are presented in **Table 1**. The patterns among both muscle types were
 350 similar when represented this way. The largest differences observed were between O₂
 351 and N₂ conditions and were not substantially different between fed and fasted groups.
 352 Interestingly, the smallest difference in glycogen concentration observed was in the fasted
 353 O₂ condition for each muscle. This observation indicates the use of alternative (oxygen
 354 dependent) fuel sources.

355

356 **Table 2: Tissue glycogen concentrations in EDL and soleus muscles of fed V. fasted mice**
 357 **following O₂ or N₂ protocols.**

Tissue	Group	Condition	Glycogen(nmol/mg)	StDev(nmol/mg)	R _{Glyc} (%)
EDL	Fed	O ₂	22.4†	4.4	-34.8
		N ₂	7.9†	5.1	-77.0
	Fasted	O ₂	18.8†	7.3	-2.5
		N ₂	7.6†	1.3	-60.6
Soleus	Fed	O ₂	42.1†*	6.6	-31.9
		N ₂	29.0†*	1.6	-53.1
	Fasted	O ₂	23.5†*	8.8	-8.9
		N ₂	9.8†*	4.2	-62.0

358 Units are nanomoles hydrolyzed glucosyl units/milligram tissue wet weight (nmol/mg). The
 359 Response Coefficient (R_{Glyc}⁻) indicates the percent change relative to the baseline group means

360 (Presented in Table 1). Sample standard deviation (StDev). * $p < .05$ Fed V. Fasted Groups. † $p < .05$
361 O₂ V. N₂ Groups. N=4.

362

363 Total adenosine nucleotide (TAN) concentrations were examined as a measure of the
364 aggregate tissue energetic state at the end of each protocol. Reductions in the
365 concentrations of the total adenosine nucleotide pool, and accumulation of IMP, are
366 measures of the muscles' inability to resynthesize ATP(27). Following the O₂ protocol, the
367 TAN pool decreased slightly in both EDL (**Fig 4C**) and soleus (**Fig 4D**) muscles compared
368 to our reported baseline values (**Fig 4 A,B**), but did not differ between fed and fasted
369 groups. Following N₂ protocols, there were large decreases in the TAN pool in both the
370 EDL (**Fig 4E**) and soleus (**Fig 4F**), with accompanying increases in the tissue IMP
371 concentrations (**Fig 4G,H**). However, no substantial differences were observed between
372 the fed and fasted groups for either muscle type.

373

374 **Fig 4: Metabolic characteristics of isolated EDL and soleus muscles under limiting**
375 **conditions of resting glycogen and oxygen availability.** Total adenosine nucleotide
376 concentrations at baseline for the EDL (A) and soleus (B). TAN concentrations following
377 the 180 min. protocol in 95% O₂ for the EDL (C) and soleus (D). TAN concentrations
378 following the 180 min. protocol in 95% N₂ (E,F). IMP accumulation after each protocol,
379 compared across all three conditions for the EDL (G) and soleus (H).
380 N=4/treatment/group. Data are presented as mean \pm SD. * $p \leq .05$ O₂ V. N₂ (V. Basal)
381 Groups.

382

383 Previous reports have indicated that dystrophin IF staining is rapidly reduced in skeletal
384 and cardiac muscle during early myonecrosis(19,32). Immunofluorescent staining for the

385 sarcolemmal protein dystrophin and the extracellular matrix protein laminin was performed
386 on a subset of transverse sectioned muscles to assess the possibility that muscles were
387 incurring damage during the contraction protocols. No apparent changes were observed
388 in the EDL (**Fig 5A**) or soleus (**Fig 5C**) under O₂ or N₂ conditions, indicating that the muscle
389 tissue remained intact during the experiments. Degradation of myofibrillar structures are
390 another well characterized indicator of myonecrosis development(33). Parallel
391 assessments were made to accompany the dystrophin/laminin stain. Fibrous actin was
392 stained in fixed whole mount muscle specimens utilizing optical sectioning to assess the
393 intramyofibrillar-IMF and perinuclear-PN regions of the myofibers at baseline and following
394 the N₂ protocol in the EDL (**Fig 5B**) and soleus (**Fig 5D**). Together these assessments did
395 not reveal any qualitative indication of damage.

396

397 **Fig 5: Assessment of structural integrity of the muscles following experimental**
398 **protocols.** To control for the possibility that the muscles were structurally damaged during
399 the contraction protocols, we performed immunofluorescence against sarcolemmal and
400 extracellular matrix proteins. Image panels of dystrophin (green), and laminin (red) stained
401 transverse EDL (A) and soleus (C) muscle sections under each of the conditions tested.
402 Sarcomeric actin was stained using phalloidin (Cyan) in fixed/permeabilized whole mount
403 muscles at baseline or following 180 mins of severe hypoxia (95% N₂); EDL (B) and soleus
404 (D). Optical sectioning facilitated imaging in the intra-myofibrillar (IMF) and perinuclear
405 (PN) regions of the muscle fibers. Scale bars are 1000µm (A, B Left Panel), 200 µm (A,B
406 right panels), and 25 µm (B,D).

407

408

409 **Discussion**

410 Skeletal muscle is among the most metabolically dynamic tissues in the body, and is
411 capable of sustaining a 100-fold change in ATP utilization rate during contraction(34). The
412 total cost of ATP during contraction is proportional to the duration, intensity, and type (i.e.
413 shortening vs. non-shortening)(35). Glycogen is the primary storage form of glucose in
414 skeletal muscle, and is a major source of fuel during most forms of muscle activity(36).
415 Importantly, glycogen is also the primary source of stored fuel utilized to regenerate ATP
416 via substrate level phosphorylation in anaerobic glycolysis during severe hypoxia(35).
417 Depletion of stored muscle glycogen by fasting or exhaustive exercise results in impaired
418 fatigue resistance and recovery in isolated rodent muscles under normoxic conditions(36–
419 38). Under ischemic conditions, this effect would be expected to lead to cumulative
420 reductions in energetic capacity due to the inability to resynthesize ATP and phospho-
421 creatine (PCr) that is used in support of contraction or resting metabolic processes.
422 Overnight fasting in rodents results in more dramatic metabolic effects than human
423 overnight fasting, but induces experimentally reproducible reductions in systemic
424 carbohydrate stores that are similar to more extreme physiological conditions such as
425 hyperinsulinemia, hypoglycemia, or post exercise recovery(11,14,23,39). We were
426 somewhat surprised to find that dramatically reduced muscle glycogen levels had no
427 substantial effect on the timing or magnitude of muscle functional impairment under
428 ischemic conditions. Our findings indicate that both muscle types retain a large pool of
429 stored glycogen that is non-essential for reserve mechanical force production during
430 hypoxia. It is not clear what the reserve glycogen pool contributes to *in vivo* during fasting.
431 Future studies could be directed to investigate its' potential involvement in the
432 maintenance of systemic glucose homeostasis through the production of free amino acids
433 (i.e. alanine and glutamine) or lactate which can be converted to glucose in the
434 liver(40,41).

435

436 In mouse EDL and soleus muscles, as much as 50% of the resting metabolic rate has
437 been attributed to maintenance of intracellular calcium homeostasis(42). We observed a
438 rapid increase in passive tension development in the fasted group relative to the fed group
439 under N₂ conditions in both muscle types. The observed increase in the rate of passive
440 tension development was the only measurement that was substantially different between
441 the fed and fasted groups. This phenomenon is most likely indicative of progressive
442 impairment of calcium handling as the capacity for ATP re-synthesis was gradually
443 depleted(27). This effect may have implications for reperfusion timing, as it has been noted
444 that calcium handling impairment prior to reperfusion is associated with poor salvage
445 outcomes(4,43).

446 The dynamic requirements of ATP during muscle contraction require a similarly dynamic
447 supply of carbon fuel sources that are derived from both blood and intracellular stores.
448 Physiological and anatomical adaptations (i.e. capillary density, size of stored energy
449 substrate pools, and mitochondrial density/function) are known to facilitate large
450 differences in capacity for spontaneous vs. sustained exercise in different species(44).
451 Similar adaptive differences are highlighted in the distinct microanatomical differences of
452 mouse locomotory EDL and postural soleus muscles. These adaptive differences,
453 combined with their similar size and relatively homogeneous fiber type compositions,
454 make these muscles excellent candidates for comparative studies of muscle energy
455 metabolism.

456 Skeletal muscle fiber types are categorized by a range of intrinsic metabolic and
457 mechanical properties(45). Human muscles are generally of mixed fiber type, but mouse
458 muscles consist of more homogeneous fiber type distributions, making them a practical
459 model for studying fiber type specific effects (soleus: 1:1 slow type I/fast type IIa; EDL: 9:1
460 fast type IIb/fast type IIa)(29,46). At face value, it may seem intuitive that fast glycolytic

461 fiber types would be better suited to performance during hypoxia due to their preference
462 for stored carbohydrate dependent energy metabolism(36,37,47). However, several
463 studies have indicated a high degree of sensitivity of fast glycolytic muscles to
464 ischemia/reperfusion injury(16,17,48). One important contributing factor to this effect is an
465 energetic inefficiency of contraction due to interactions at the level of the acto-myosin
466 crossbridges(28,49). In the present study, we observed that Soleus muscles stored more
467 glycogen at baseline, had greater specific force/work capacities, and produced absolute
468 force for a longer period during ischemia compared to EDL muscles. These observations
469 are consistent with previous reports(48,50). Though the absolute differences in glycogen
470 concentrations between groups were larger in the soleus compared to the EDL, the
471 response coefficient (R_{Glyc}) which facilitates interpretation of group differences relative to
472 their baseline concentration, indicated that the patterns of utilization were not different
473 between the two types of muscles. We interpret these findings to mean that the greater
474 basal glycogen concentration observed in the soleus muscles was likely not the primary
475 factor underlying it's enhanced ischemic mechanical performance.

476

477 **Conclusion**

478 Investigating the key factors that affect the timing of muscle energetic failure during
479 ischemia will aid in identifying optimal windows for therapeutic intervention. We predicted
480 that the amount of stored carbohydrate is one such factor, as it is a major contributor to
481 anaerobic energy metabolism and is influenced by several physiologically relevant
482 conditions. We conclude that mouse hindlimb muscles maintain a large pool of stored
483 carbohydrate that is utilized during fasting but does not contribute substantially to the
484 timing of functional decline during acute ischemia. The carbohydrate lowering effects of
485 fasting did not substantially affect the total capacity or timing of contractile function
486 impairment in either muscle type. However, fasting did result in substantial increases in

487 early passive tension development, which may have implications for the timing of
488 reperfusion or therapeutic administration. We also found that soleus muscles maintained
489 a greater total force capacity and became impaired more slowly than EDL muscles,
490 independent of glycogen utilization during the experimental period. This finding supports
491 several previous observations and bolsters the notion that susceptibility to acute ischemic
492 injury is not uniform across muscle types.

493

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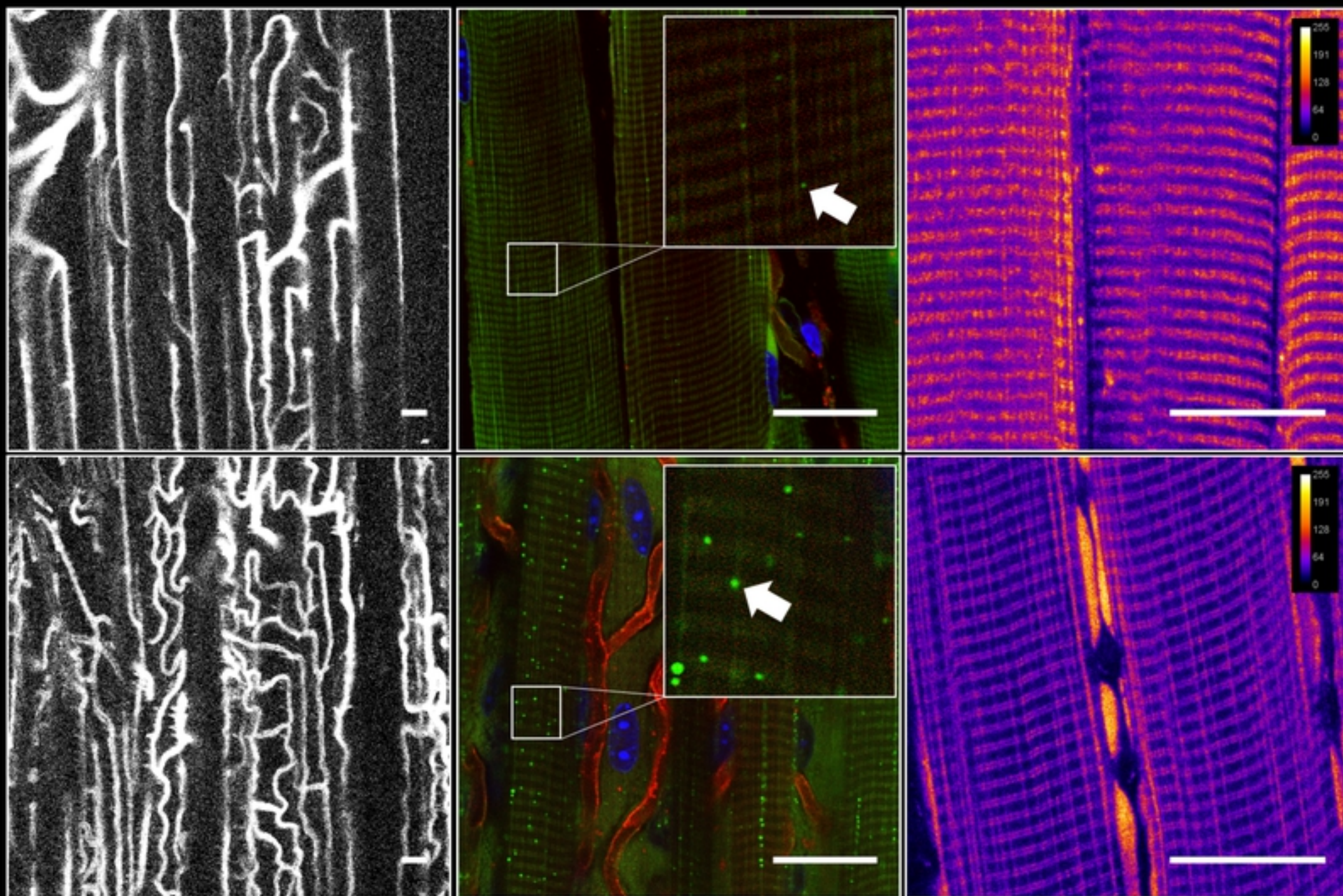
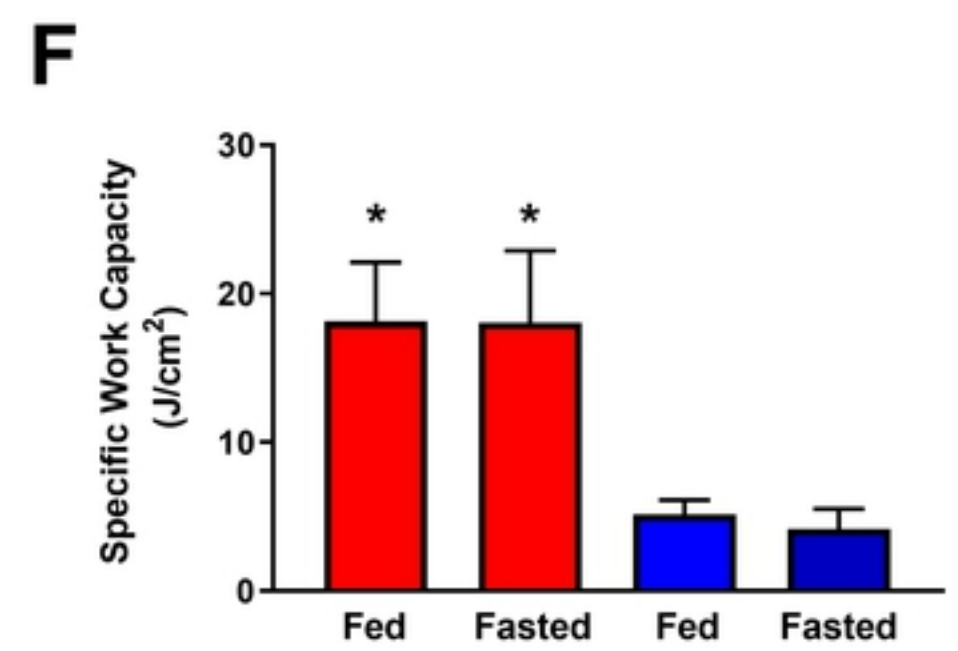
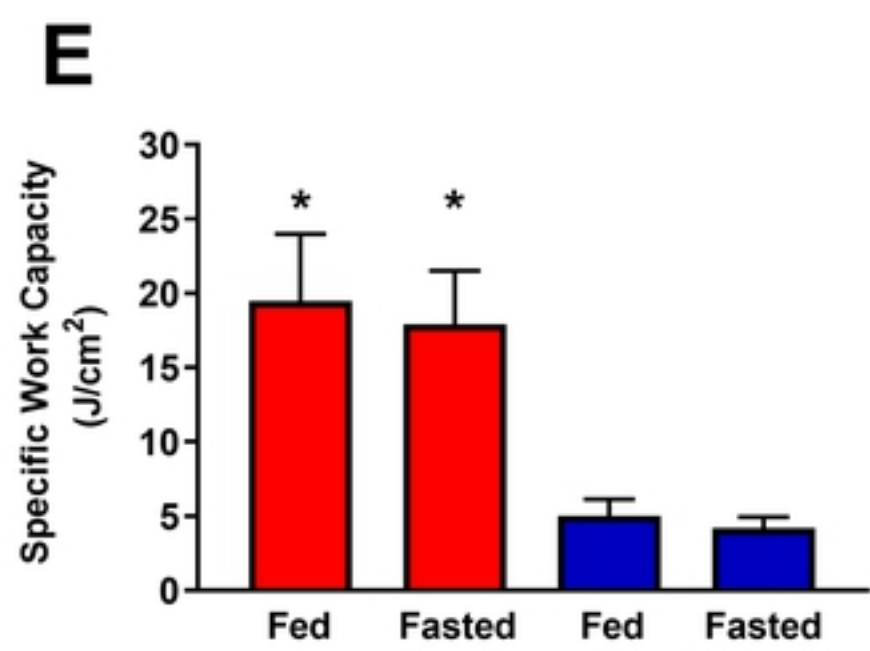
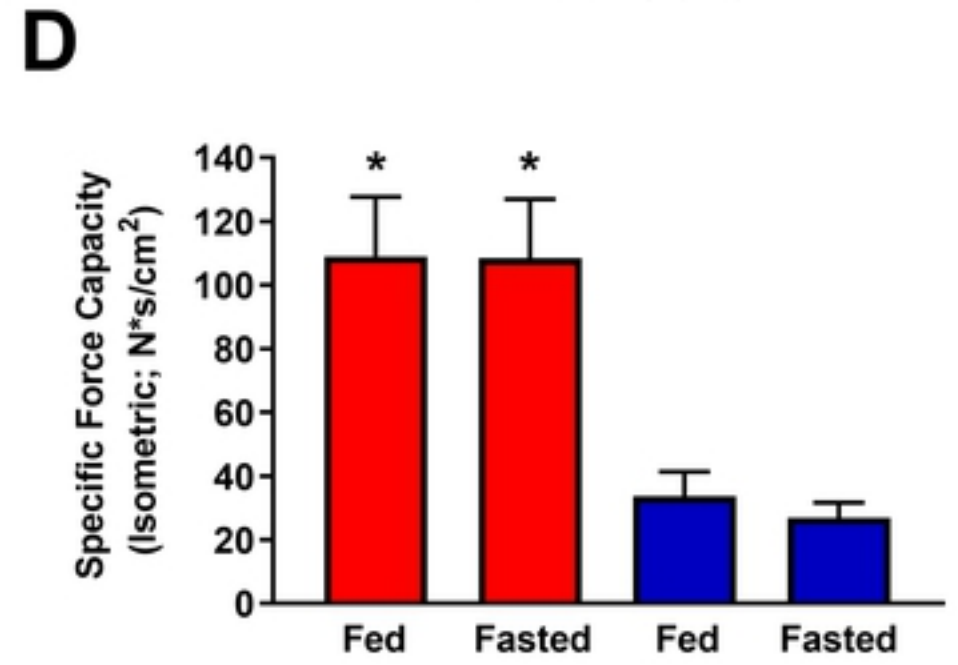
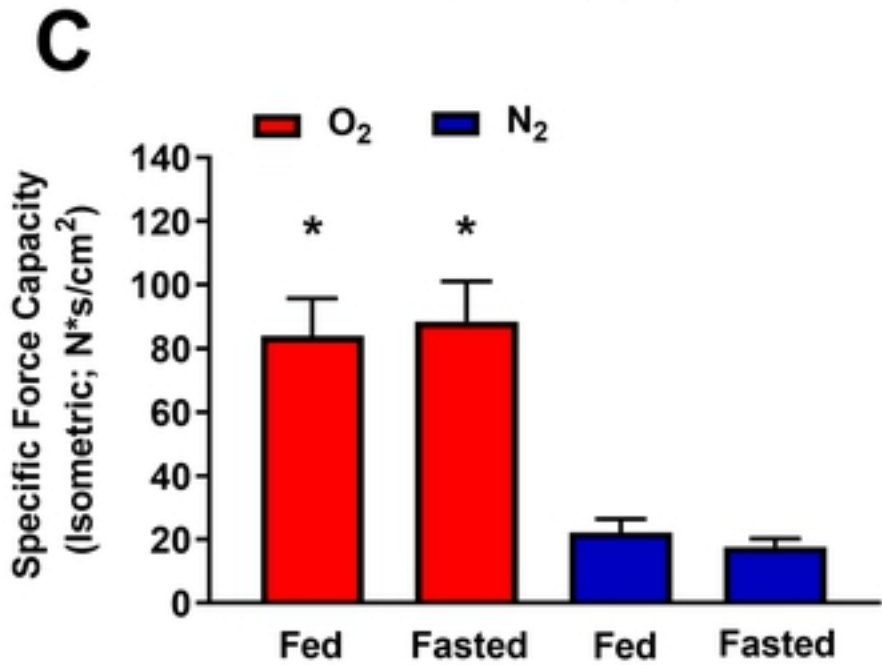
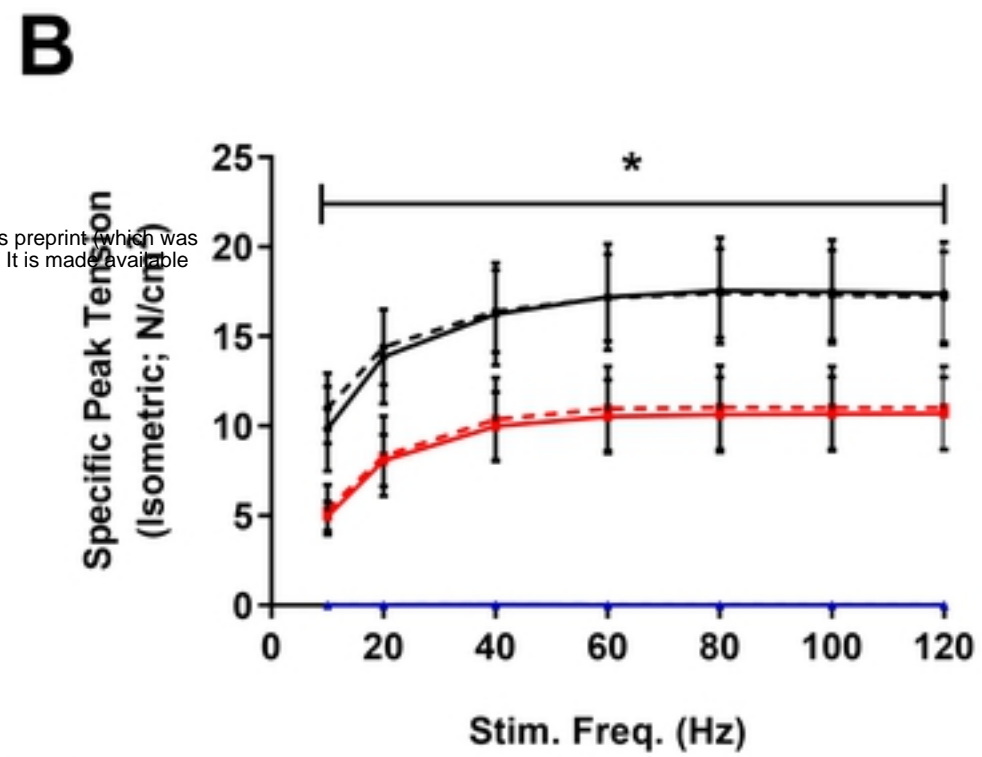
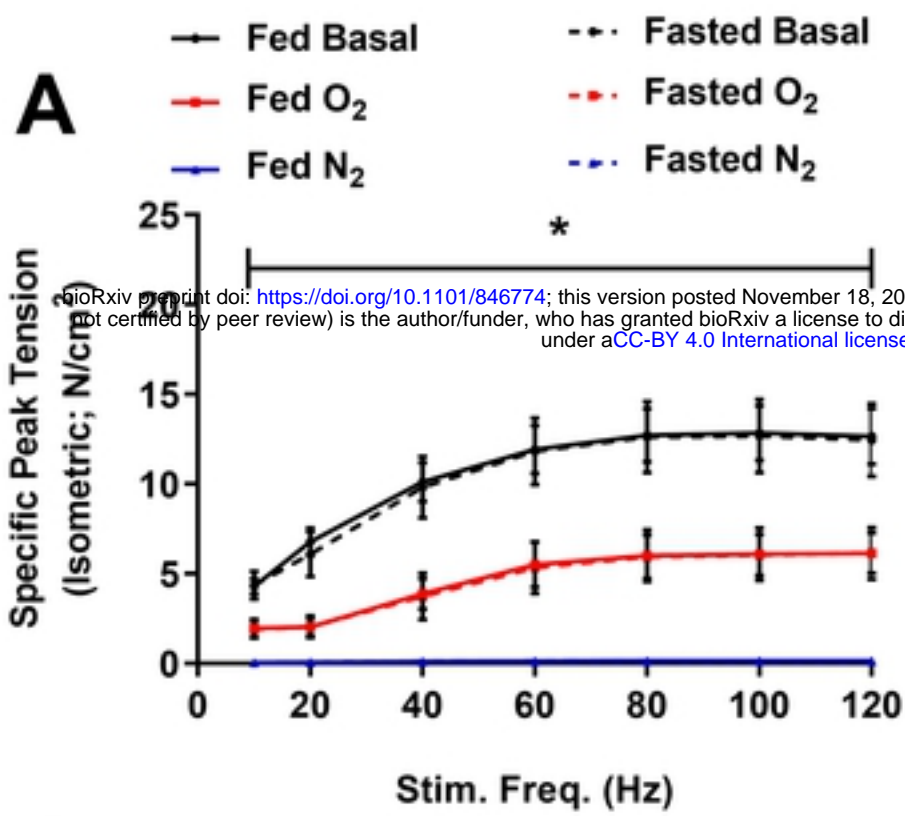


Figure 1

EDL**Soleus**

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Figure 2

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Soleus

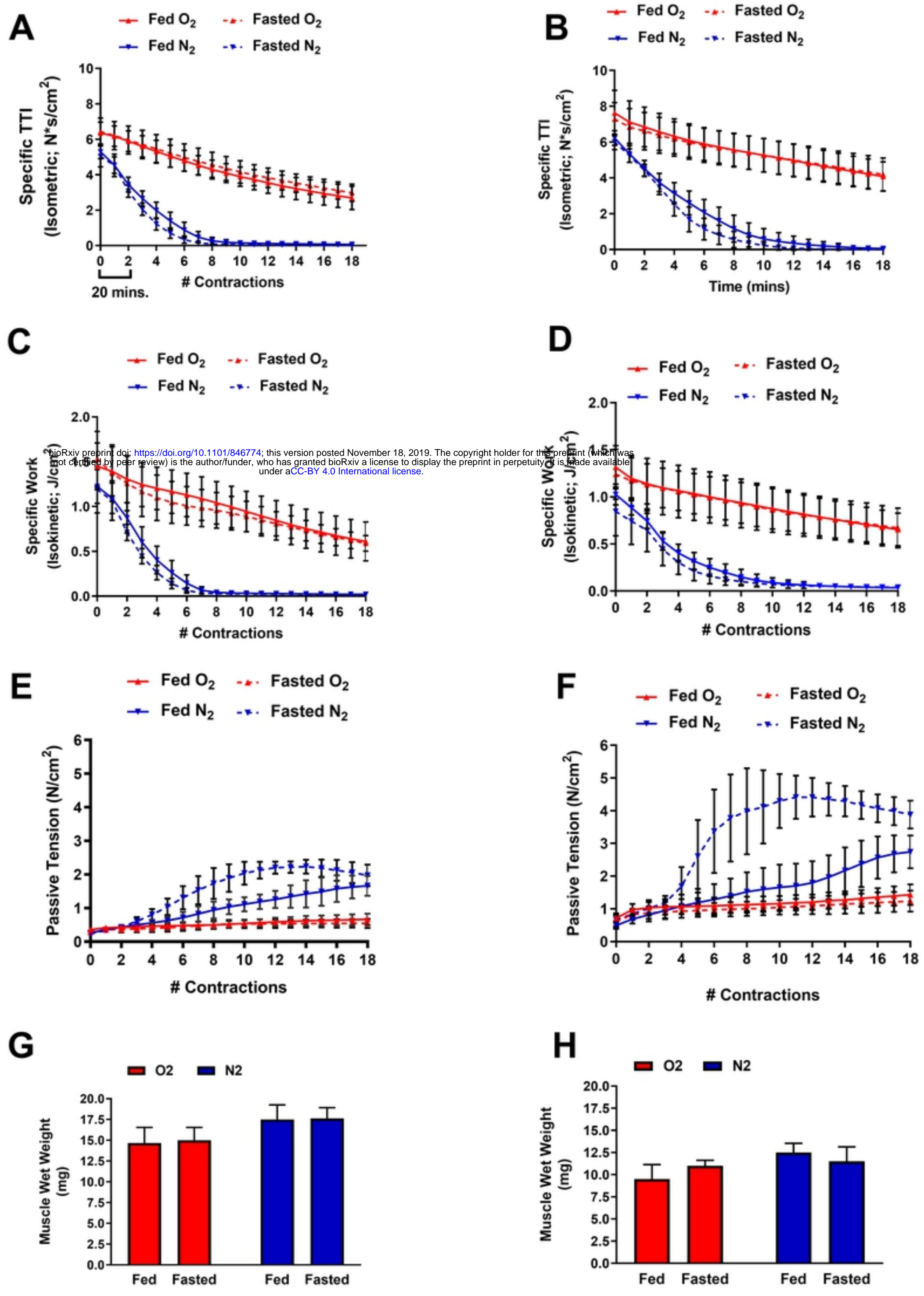
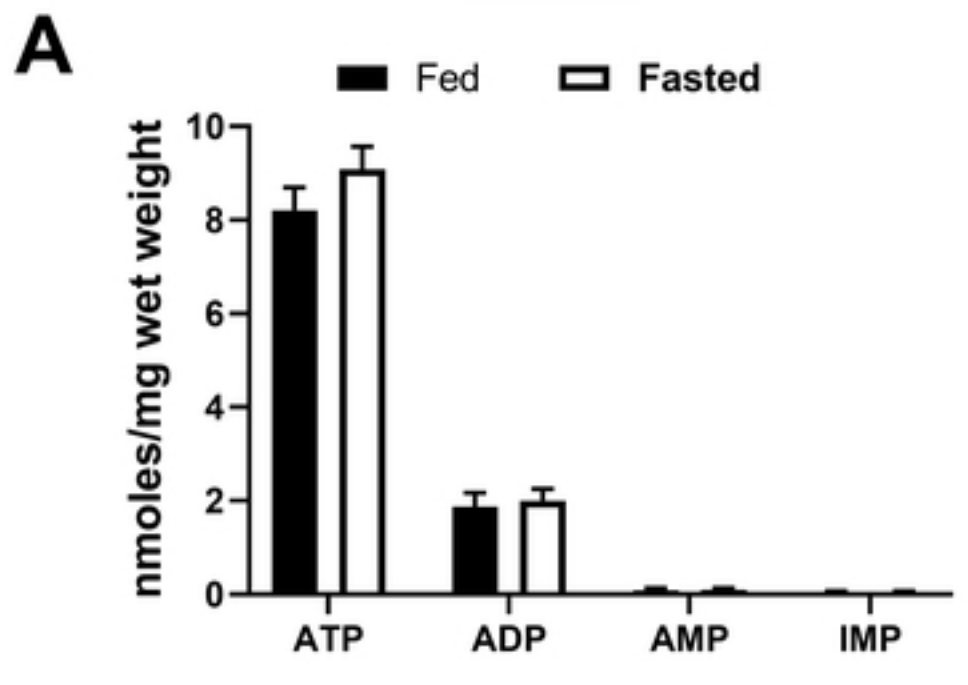


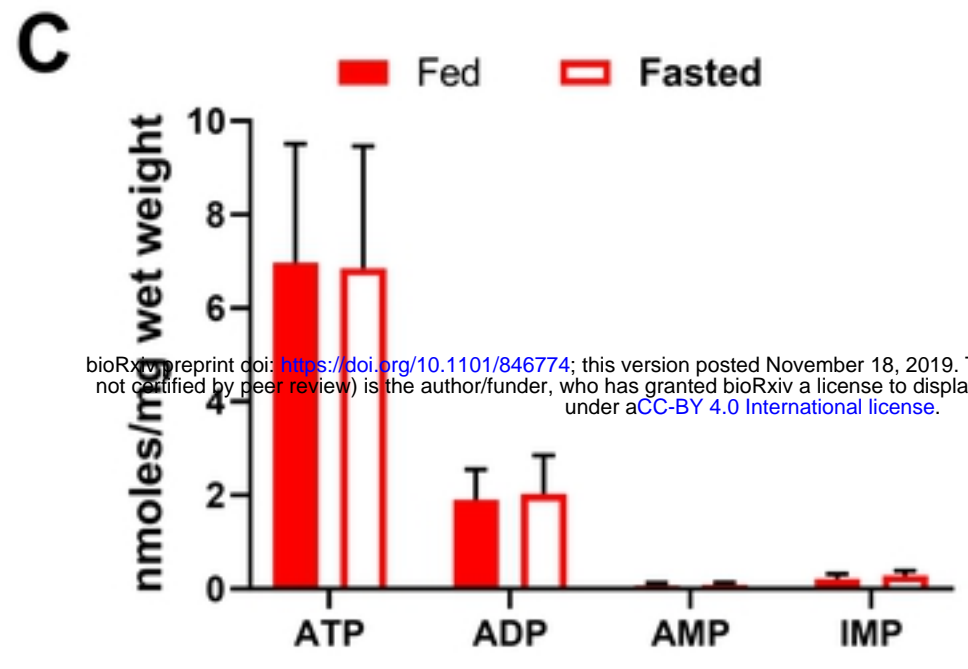
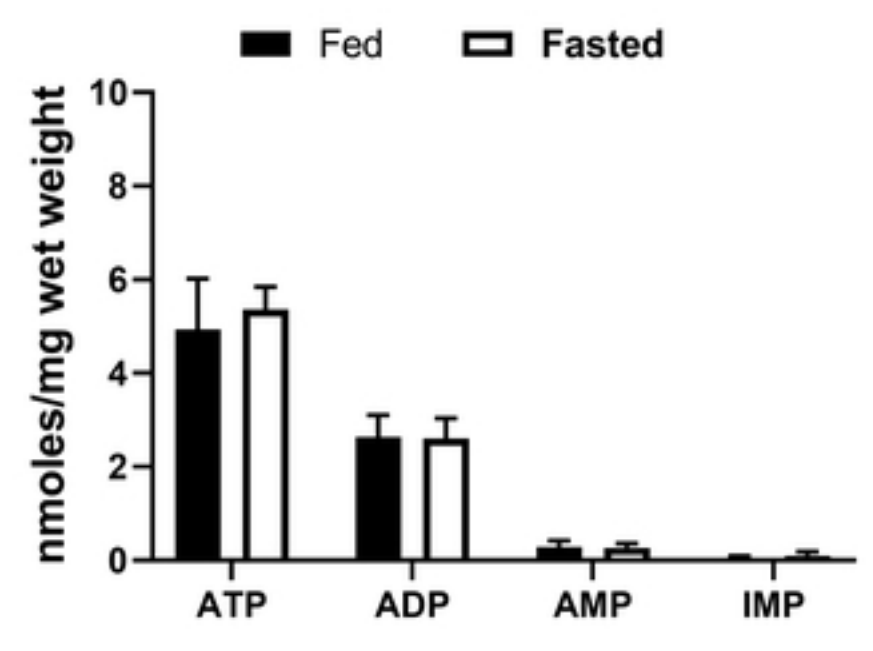
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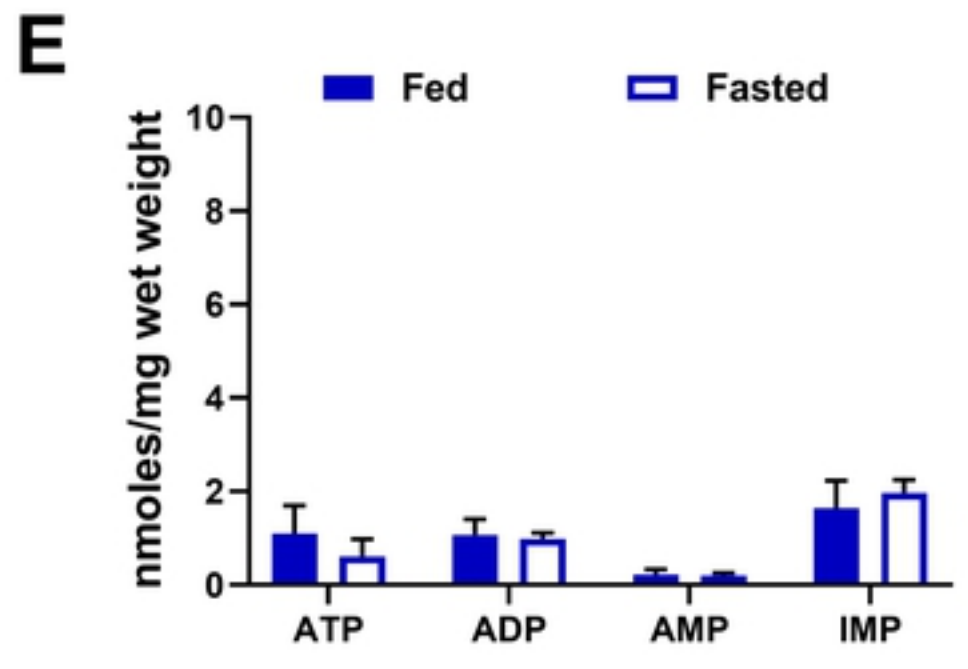
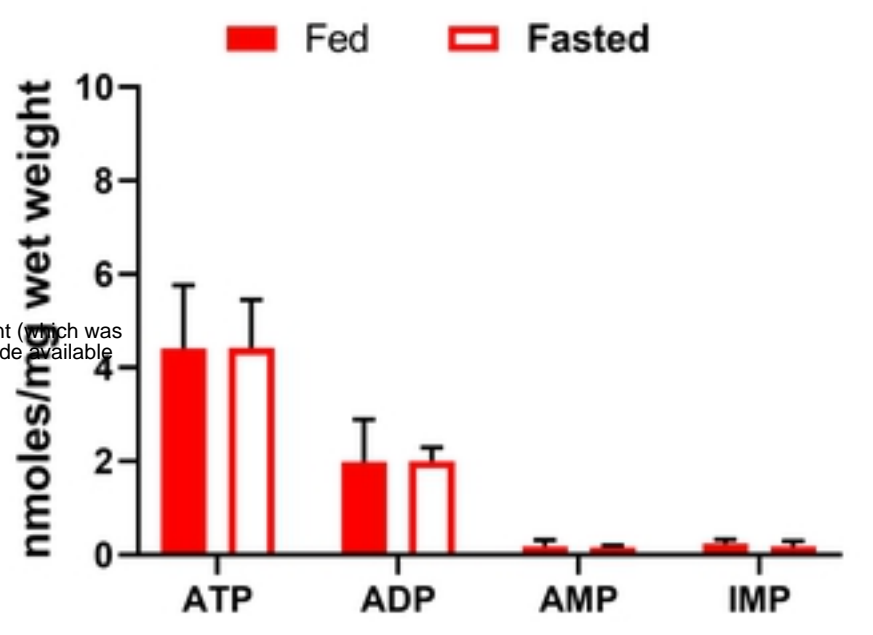
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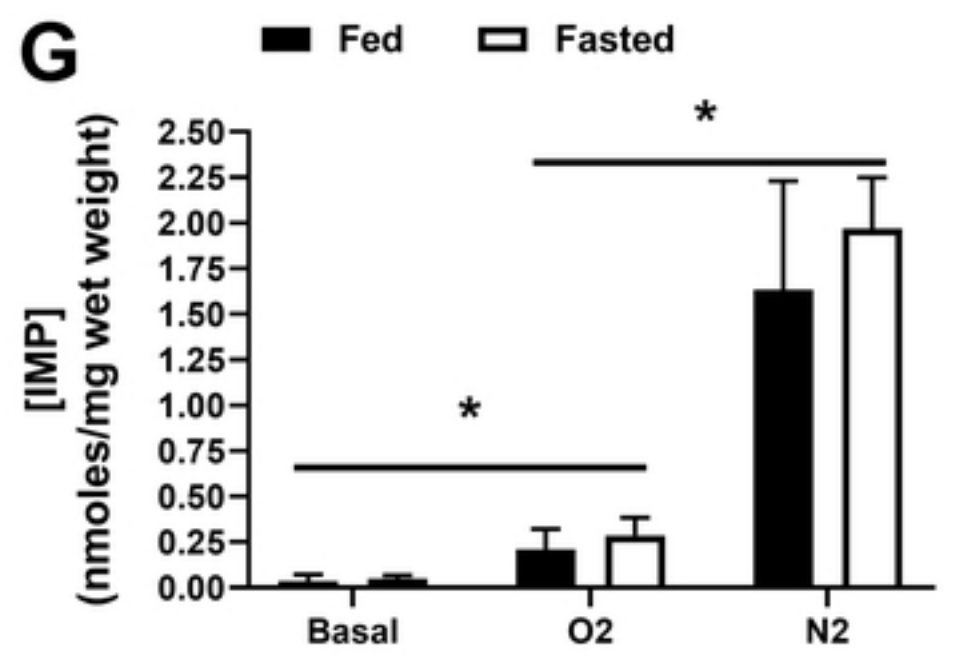
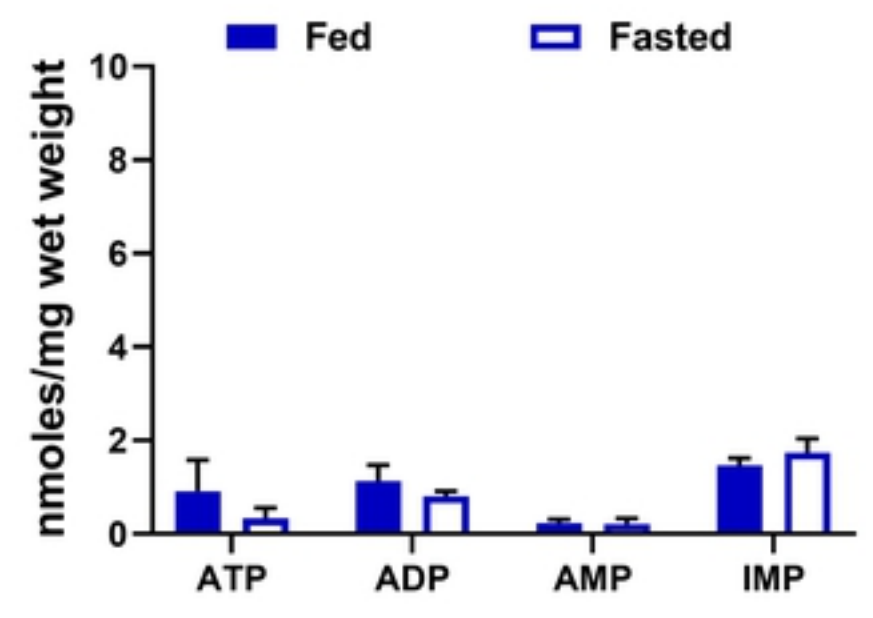
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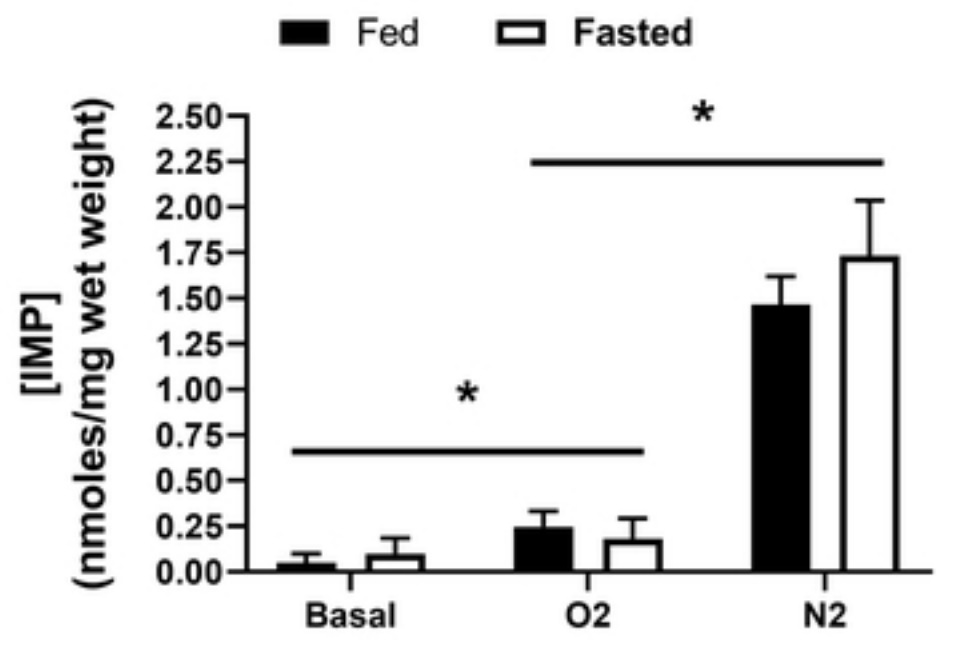


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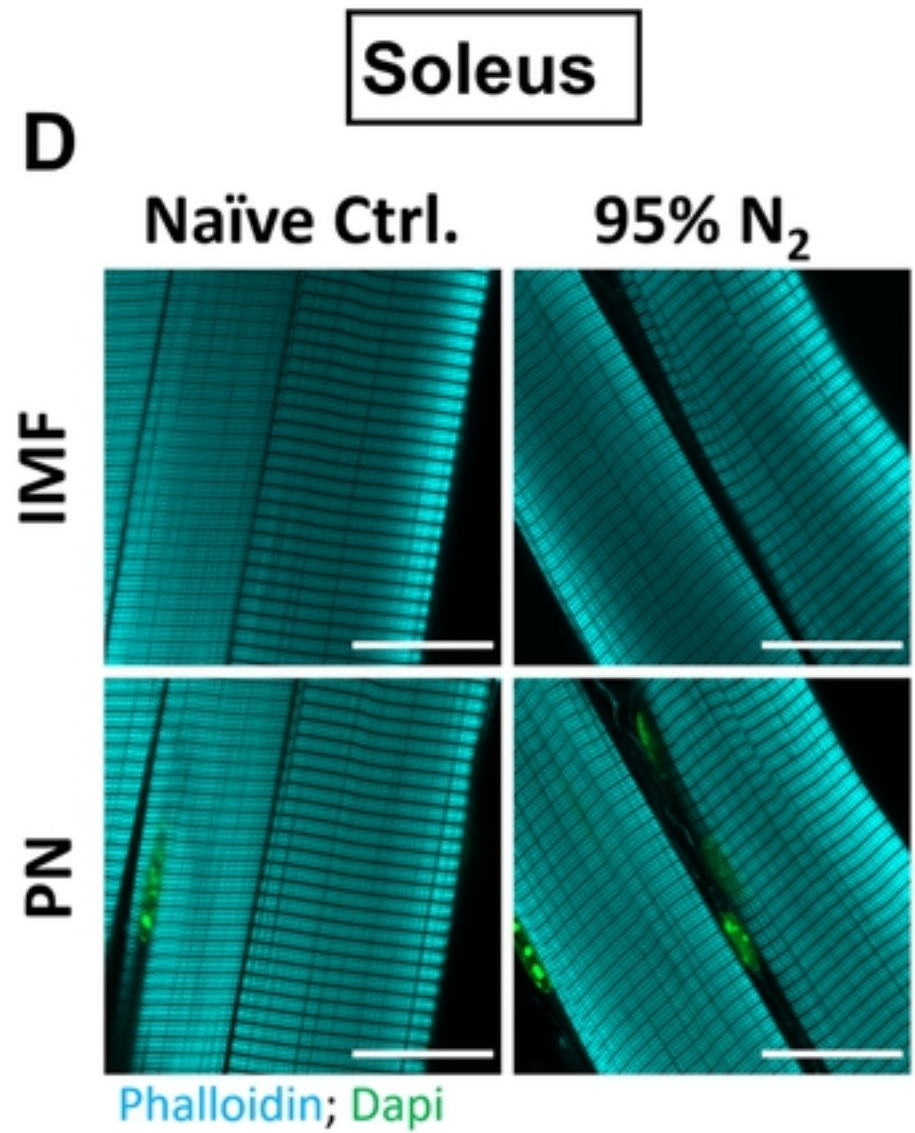
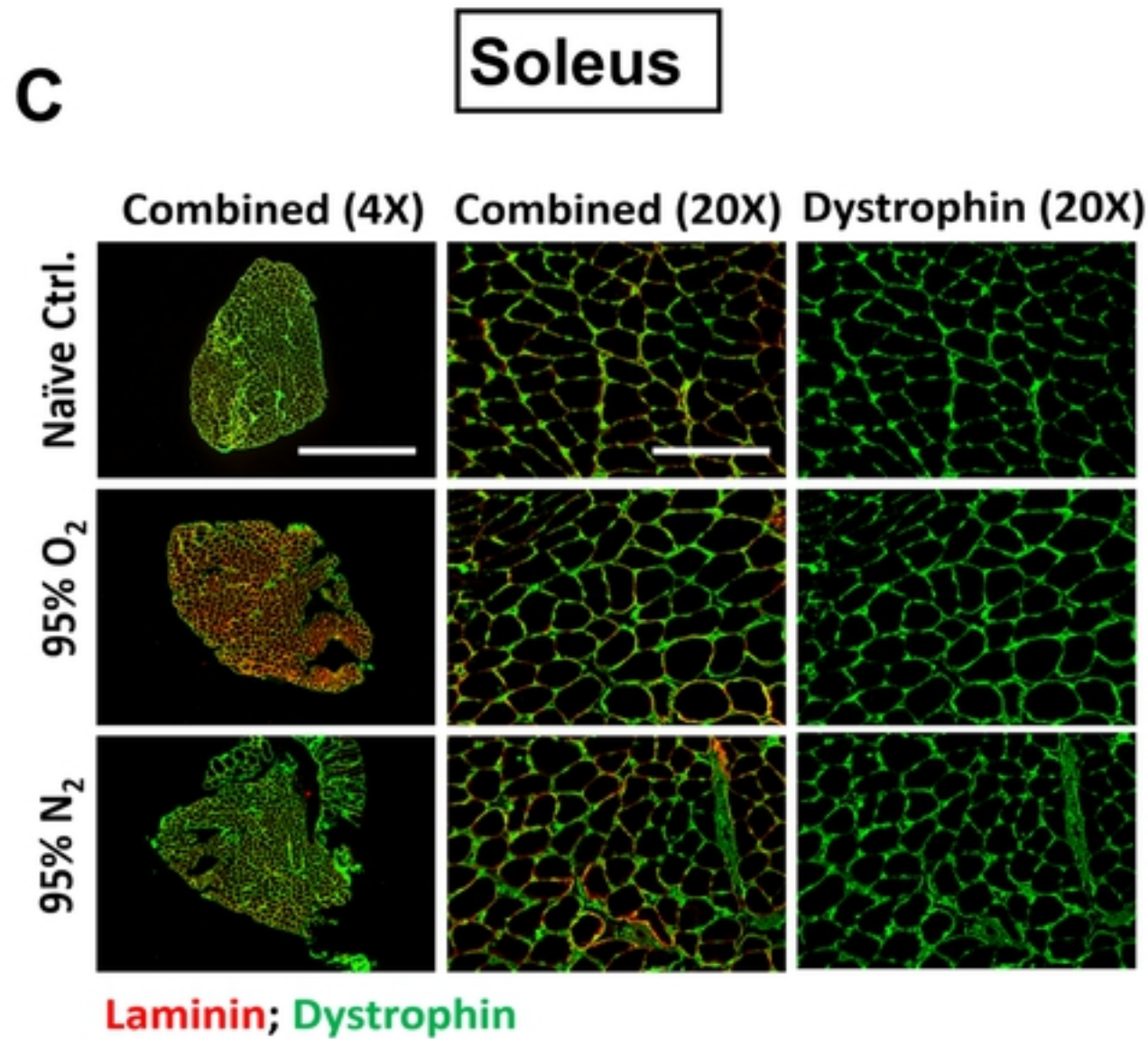
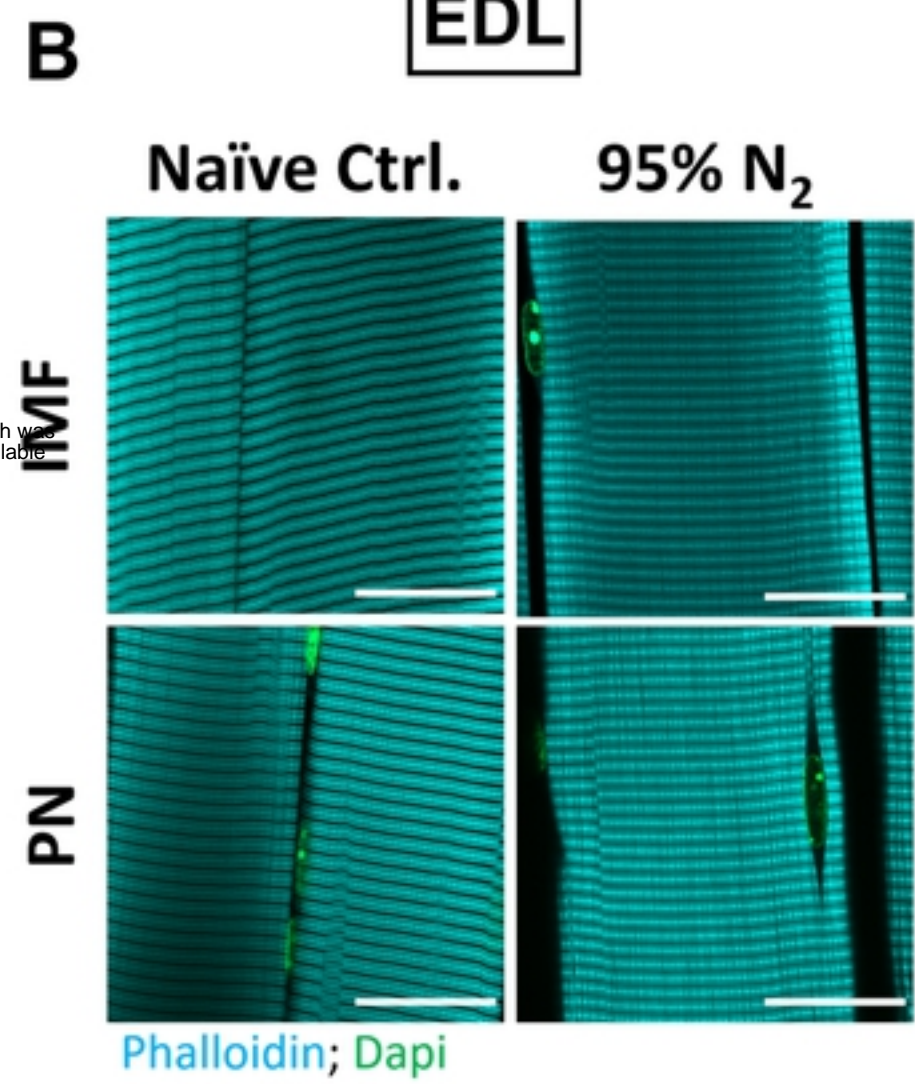
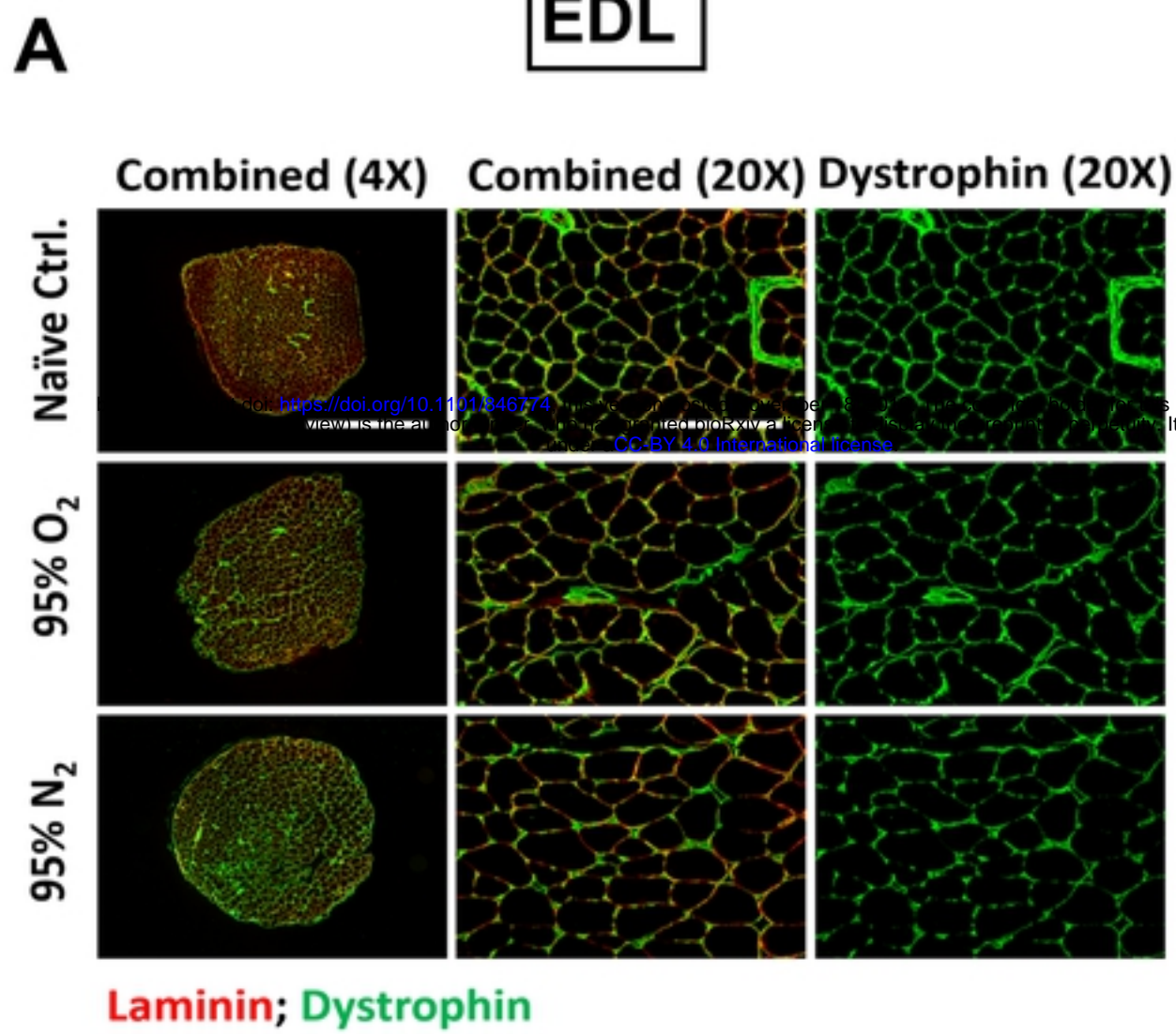


Figure 5