# Mitochondrial uncoupler BAM15 improves skeletal muscle function and mitochondrial respiration in Sarcopenia

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**Abstract**

**Background:** Ageing is accompanied by progressive declines in skeletal muscle mass and strength, culminating in sarcopenia, a condition that contributes to frailty, multimorbidity, and mortality. Age-related changes to mitochondria lead to oxidative damage and dysfunction and are proposed to occur early in the trajectory of sarcopenia, supporting the candidacy of mitochondrial-protective therapies. Here, we test the efficacy of mitochondrial uncoupler BAM15 in age-dependent sarcopenic mouse models.

**Methods:** Male and female MitoQC mice aged 24 months received either standard chow or chow supplemented with BAM15 (0.033% mg/g) ad libitum for eight weeks (n=13–14/group). Young (3-month-old) mice served as reference controls (n=8/group). Muscle mitochondrial respiration was assessed in permeabilized fibres, and contractile function was measured in isolated extensor digitorum longus and soleus muscles. Mitophagy was quantified by immunofluorescence confocal microscopy. Data were analyzed using one- or two-way ANOVA followed by Dunnett's or Bonferroni's multiple comparison tests.

**Results:** Aged male and female mice exhibited reduced gastrocnemius muscle mass relative to body mass compared with young controls ($p<0.05$; ~18% and ~32% loss, respectively). BAM15 did not alter muscle size but reversed the age-related loss of contractile function in EDL muscles, to that of the young reference controls in both sexes ($p<0.05$; ~33% in males, ~16% in females). In male mice, BAM15 improved mitochondrial efficiency, evidenced by restoration of Complex I–linked respiration and decreased proton leak (~52% improvement; $p<0.05$), and normalized protein levels of oxidative stress marker 4-HNE, without changes in mitophagy or mitochondrial content. In females, BAM15 did not improve mitochondrial parameters, which may be, in part, due to aged female muscle exhibiting unchanged Complex I leak and 4-HNE protein abundance, alongside lower complex I subunit (NDUFB8) protein abundance.

**Conclusions:** BAM15 improved skeletal muscle mitochondrial efficiency and contractile function in aged male mice, supporting the potential of mitochondrial uncoupling as a therapeutic strategy for sarcopenia.

**Keywords**

Sarcopenia, mitochondrial uncouplers, skeletal muscle dysfunction, mitochondrial respiration, oxidative stress.

## Introduction

Sarcopenia is the clinical term for age-related skeletal muscle decline characterized by loss of muscle mass, weakness and reduced physical capacity [1]. This culminates into frailty conditions which are associated with increased risk of falls and fractures, hospitalization, health care costs, co-morbidities and mortality [2]. To date, therapeutic interventions for the treatment of sarcopenia are limited, and those proposed often fail to show functional improvements in physical capacity [3]. Therefore, novel strategies to protect against age-related muscle decline are warranted.

Mitochondrial dysfunction is a hallmark of ageing with several mechanisms proposed [4]. Accordingly, mitochondrial uncoupler compounds are being explored as potential interventions to delay and prevent sarcopenia progression [5-7]. Mitochondria provide cellular energy for biological processes, primarily through electron transport chain (ETC) oxidative phosphorylation This process pumps protons from the mitochondrial matrix into the intermembrane space to create the proton motive force (PMF) that couples substrate oxidation to ATP synthesis [8]. In ageing, compromised ETC functional capacity leads to elevated PMF and increased reactive oxygen species (ROS) production. Mitochondrial uncoupler compounds lower the PMF and decreases ROS production by transporting protons from the mitochondrial intermembrane space into the mitochondrial matrix [5, 6].

Here, we evaluated the mitochondrial uncoupler BAM15 as a therapeutic for sarcopenia. BAM15 is a mitochondrial-targeted protonophore ((2-fluorophenyl){6-[(2-fluorophenyl)amino](1,2,5-oxadiazolo[3,4-e]pyrazin-5-yl)}amine) that has been shown to prevent diet-induced obesity, insulin resistance [9] and skeletal muscle decline in sarcopenic obesity mouse models [10]. Unlike traditional uncouplers such as carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) [11], BAM15 does not induce mitochondrial membrane depolarization or damage and shows excellent bioavailability in skeletal muscle following oral delivery [9]. We hypothesised that mild mitochondrial uncoupling via BAM15 supplementation would preserve skeletal muscle mitochondrial function and thereby protect against age-associated muscle decline.

## Methods

### Experimental protocols and treatments:

*Mouse cohort*

All animal experiments were conducted in compliance with Garvan Ethics Committee guidelines (21/20 protocols) and in accordance with the Australian code of practice for the care and use of animals for scientific purposes. Mitophagy reporter mice (MitoQC) [12] were kindly provided by Professor Ian Ganley (University of Dundee, UK). The MitoQC construct is a constitutive knockin of tandem fluorophore tag mCherry and GFP on outer mitochondrial membrane protein FIS1 [101–153]. MitoQC colony breeding and maintenance were completed at Australian Bioresources (Moss Vale, NSW, Australia) and genotyping was performed by Garvan Molecular Genetics (Darlinghurst, NSW, Australia). Mice were transferred to the Biological Testing Facility at Garvan Institute of Medical Research and were housed on a 12-hour light/dark cycle with ad libitum access to standard rodent chow and water. Once MitoQC mice were assigned to the aging colony and became 12-months of age they received bi-weekly health-checks and regular welfare monitoring by researchers and animal technicians until they reached the desired experimental age, i.e., 24-months-old.

*Study design*

At 24 months of age, male and female MitoQC mice were randomly allocated to an 8-week diet-intervention of standard chow (26M CHOW) or BAM15 supplemented chow (26M BAM15; (0.033% (mg/g)), with $n$=14/group. The age range of 24–26 months in the C57BL/6J mouse strain corresponds with established sarcopenia model classifications, characterized by loss of muscle mass and function [13]. These mice received daily welfare monitoring alongside tri-weekly recording of body weight and food intake. The dosing strategy for BAM15 was adapted from our previous work in C57BL/6J mice [9]. 1 male 26M CHOW and 1 female 26M BAM15 supplemented mice were euthanised during treatment due to reaching ethical humane endpoints and were excluded from analysis. Untreated 3-month-old male and female MitoQC mice (3M CON: $n$=8/group) were used as a healthy reference control group. Power calculations were based on our previous publication [14], with a minimum of n=8 aged mice per group was estimated to be sufficient. To accommodate age-related attrition, group sizes were increased by ~50%, consistent with reported survival rates from the Jackson Laboratory of C57BL/6 mice by 28 months of age.

*BAM15 Diet Preparation*

BAM15 was prepared as previously described [15]. Briefly, standard chow was first powdered using a food processor and used for all treatment groups. Powdered chow was then mixed with the correct concentration of BAM15 and compressed into pellets using a small pellet mill (Gemco ZLSP-120B). For the standard chow diet, powdered diet was pressed into pellets without adding any drug.

*Tissue collection and processing*

At study end, mice were anaesthetized using isoflurane (5% induction and 2-3% maintenance). Muscles and organs were surgically removed, weighed and collected via snap-freezing in liquid nitrogen or immersive fixation (3.7% PFA in 200 mM HEPES, pH 7). Exceptions include: micro-surgical harvesting of EDL and soleus (SOL) muscles for contractility testing and collection of one gastrocnemius (GTN) muscle per mouse which was immediately incubated in ice-cold BIOPS buffer post-harvest (2.77 mM CaK2EGTA, 7.23 mM K2EGTA, 5.77 mM Na2ATP, 6.56 mM MgCl2-6H2O, 20 mM taurine, 15 mM Na2-phosphocreatine, 20 mM imidazole, 0.5 mM dithiothreitol, 50 mM MES hydrate, pH 7.1). Euthanasia was confirmed following cardiac removal.

*Skeletal muscle contractile function*

*Ex vivo* electrophysical testing of skeletal muscle contractility was conducted on EDL and SOL muscles as previously described [16]. Muscles were tied at both tendons with 4.0 surgical silk thread before being excised from the hindlimb and placed into individual organ baths of a Myodynamics Muscle Strip Myograph System (DMT). Each organ bath was filled with a modified Krebs-Henseleit solution (NaCl 118 mM, MgSO4·7H2O 1 mM, KCl 4.75 mM, KH2PO4 1 mM, CaCl2 2.5 mM, NaHCO3 24 mM and glucose 11 mM; pH 7.4), bubbled with carbogen (5% CO2 in O2) and maintained at 30°C. The optimal length of each muscle was established through the delivery of sequential twitch contractions, while incrementally stretching each muscle, until the point where the greatest twitch force produced was determined and the resting length was measured via the micromanipulator. Next, a force–frequency protocol was performed utilizing tetanic stimulations with a square wave of 0.2ms pulses, and a pulse train duration of 350ms and 500ms for the EDL and SOL, respectively. EDL and SOL muscles were stimulated at an incremental range of frequencies from 10-150 Hz, with a 3-min rest in between each stimulation. Specific force was calculated using equations as previously described [17]. To investigate muscle resilience, muscles underwent a three-minute fatigue protocol, with EDL muscles stimulated at 100Hz every 4s, and, SOL muscles stimulated at 80 HZ every 2s. Recovery was also assessed, with tetanic

133 stimulations occurring up to 15 mins post-fatigue protocol. Surgical silk was removed and muscles were blotted
134 before tendon-free dry mass was recorded. All data were collected and annotated using LabChart Pro version 8.0
135 software (ADInstruments).
136

137 *High-resolution respirometry*

138 *Ex vivo* mitochondrial respiration was assessed in GTN muscles by measuring oxygen consumption using an
139 Oxygraph-2k (OROBOROS Instruments) as previously published [18]. GTN muscles were mechanically
140 separated into 3-5mg fibre bundles before being permeabilized in 50 µg/µL of saponin in BIOPS buffer for 30
141 mins at 4 °C. Fibre bundles were weighed and loaded into Oxygraph-2k chambers calibrated and pre-loaded with
142 2 mL of MiRO5 buffer (0.5 mM EGTA, 3 mM MgCl2-6H2O, 60 mM lactobionic acid, 20 mM taurine, 10 mM
143 KH2PO4, 20 mM HEPES, 110 mM D-sucrose, 1 g/l bovine serum albumin, pH 7.1) at 37 °C. Oxygen
144 concentration was maintained between 150-220 µM throughout the SUIT assay. First, pyruvate (10 mM) and
145 malate (2 mM) were injected to stimulate respiration via ETC cycling at Complex I. ADP was then injected at
146 incremental concentrations of 0.1 mM, 0.175 mM, 0.25 mM, 0.5 mM, 1 mM, 2 mM, 4 mM, and 6 mM, to generate
147 a dose-response curve that depicts ADP-dependent oxidative phosphorylation. ADP sensitivity can then be
148 determined through transforming data with the Michaelis–Menten enzyme kinetics fitting model using Prism
149 (GraphPad Software, Inc.). Next, substrates glutamate (10 mM) and succinate (10 mM) were injected to assess
150 phosphorylating respiration, followed by mitochondrial uncoupler Carbonyl cyanide m-chlorophenyl hydrazone
151 (CCCP – 0.5 µM) to assess maximal ETC respiration. Lastly, Antimycin A (2.5 µM) was injected as a Complex
152 III inhibitor, which ceases mitochondrial respiration and allows for the detection of non-mitochondrial respiration.
153

154 *Mitophagy assessment via immunofluorescence*

155 Quadriceps muscles were taken out of PFA 24-48 hours post fixation and placed in cryopreservation solution
156 (30% sucrose in 1X PBS, pH 7) at 4 °C. Muscles were then placed in tissue moulds and embedded in optimal
157 cutting temperature (OCT) compound (Sakura). Longitudinal cryosections were cut at 10 µm with a NX50
158 Cryostar Epredia Cryostat and mounted in ProLong Diamond Antifade (Thermofisher). Images were acquired
159 using a Nikon C2-confocal microscope equipped with a 100X oil immersion objective. Image analysis was
160 performed on ImageJ using semi-automatic plugin *mito-QC Counter* [18] to quantify mitolysosomes by red
161 punctate counting, measuring mitolysosome size and mean channel intensities. Images were pre-processed by

162 applying a Gaussian blur filter with a radius of 1-1.5µm and regions of interest drawn around muscle fibres for
163 relative normalisation. Quantification of mitophagy was performed in 15-17 fibres per section.
164

165 *Muscle lysate preparation and immunoblotting*

166 Snap-frozen GTN muscles were prepared as previously published [18]. Briefly, GTN muscles were powdered
167 on dry ice using a Cellcrusher tissue pulverizer (Cellcrusher Ltd) and homogenised via shaking in a Precellys
168 Evolution Touch (Bertin Technologies) at 8,500 rpm for 2x 30 sec cycles in ice-cold sucrose lysis buffer (50 mM
169 tris pH 7.5; 270 mM sucrose; 1 mM EDTA; 1 mM EGTA; 1% triton X-100; 50 mM sodium fluoride (NaF); 1 mM
170 sodium orthovanadate (Na3VO4); 5 mM disodium pyrophosphate (Na2H2P2O7)) supplemented with fresh
171 cOmplete™ EDTA free protease inhibitor cocktail (Roche) and phosphatase inhibitor cocktail (Sigma-Aldrich).
172 Muscle homogenates were then centrifuged for 20 min at 14,800 rpm at 4°C to remove insoluble material and
173 produce muscle lysates. Protein concentrations were determined using the RC/DC protein assay as per
174 manufacturer's instructions (Bio-Rad). Samples were prepared by mixing equivalent protein amounts of muscle
175 lysates (30ug) in 4× Laemmli buffer (Bio-Rad) reduced in β-mercaptoethanol and boiled for 5 min at 95 °C. The
176 antibody that required an exception to this protocol was the Total OXPHOS Rodent Antibody Cocktail, where
177 samples remained unboiled per manufacturer's instructions. Samples were separated by SDS-PAGE on 4-15%
178 gradient gels (Bio-Rad) at 100 V and transferred onto nitrocellulose membranes (Bio-Rad) using a wet transfer
179 system at 100 V for 60 min. Membranes were then stained in Ponceau S (Sigma-Aldrich) to check for even protein
180 loading and transfer. Membranes were then blocked in 5% non-fat milk powder milk in tris-buffered saline with
181 0.1% Tween 20 (TBST) for 60 min followed by an overnight incubation at 4 °C with primary antibody dissolved
182 in TBST containing either 3% BSA or non-fat milk powder. Primary antibodies probed include: Total OXPHOS
183 Rodent Antibody Cocktail (1:1,000; ab110413; Abcam), Anti-NDUFB8 (1:1,000, ab192878, Abcam), Citrate
184 Synthase (1:2,500, #14309, CST) and 4 Hydroxynonenal (1:1,000, ab46545, Abcam). After overnight incubation,
185 membranes were washed 3 separate times for 5 min each in TBST and then probed with a horseradish peroxidase-
186 conjugated secondary antibody (1:10,000; anti-rabbit IgG or anti-mouse IgG; CST) in TBST for 1 hour at room
187 temperature. Following another set of 3 separate 10-min washes in TBST, images were developed using ECL
188 reagent (Millipore), captured using the ChemiDoc Touch (Bio-Rad) and quantified using ImageLab software (Bio-
189 Rad).
190

*Statistics*

Data are presented as Mean ± standard deviation and were analysed with GraphPad Prism v10. Normality of the data was assessed by Shapiro-Wilk tests. For parametric data, differences between experimental groups were determined by One-way ANOVA and Dunnet's multiple comparisons testing or Two-way ANOVA and Bonferroni's multiple comparisons testing. To detect significant differences in non-parametric data, a Kruskal-Wallis testing was performed followed by Dunn's multiple comparisons testing. Significance level was set at $p<0.05$.

## Results

### BAM15 supplementation does not impact body and muscle mass in aged mice

To evaluate the potential therapeutic efficacy of BAM15 in sarcopenia, we supplemented 24-month-old male and female MitoQC mice for 8 weeks with either standard chow or 0.033% admixed BAM15 (Figure 1A). Anthropometric endpoint measures indicated that aged male and female mice had a sarcopenia-like phenotype with increased body mass (~26% & ~39%, for males and females, respectively), and reduced quadricep mass (~22% & ~17%, for males and females, respectively) and relative body to muscle mass ratios (~22% & ~35%, for males and females, respectively), compared to 3-month-old healthy controls (Figure 1B-1D). BAM15 supplementation did not affect body and muscle mass in aged male or female mice (Figure 1B-1D). This was consistent with no changes to organ mass or food intake arising from BAM15 supplementation (Supp. Figure 1).

### BAM15 supplementation improves skeletal muscle contractile function in aged mice

In males, BAM15 supplementation protected against the age-associated loss of contractile function in EDL muscles, as represented by absolute and specific force frequency relationships similar to 3M CON (Figure 2A). BAM15 supplementation preserved EDL muscle characteristics relative to 3M CON, including; mass, optimal length, twitch force and the rate of force development, which were all reduced in the male 26M CHOW group (Supp. Table 1). BAM15 supplementation resulted in a greater resilience to fatigue in EDL muscles, with improved force production during the recovery period (Figure 2A). These findings were not reflected in male SOL data, which demonstrated lower absolute and specific force-frequency relationships, and reduced resilience to fatigue

218 with lower force production during the recovery period in all aged groups (Figure 2B). However, SOL twitch
219 characteristics demonstrated that BAM15 supplementation preserved time-to-peak, which was slower in the 26M
220 CHOW group, suggesting potential protection to myosin-ATPase functionality without overall improvement in
221 force production (Supp. Table 1). There were no additional effects on SOL twitch characteristics observed (Supp.
222 Table 1). Consistent with male data, BAM15 supplementation protected against the loss of contractile function in
223 female EDL muscles, represented by absolute and specific force frequency relationships (Figure 2C). However,
224 there were no changes to female EDL twitch characteristics from BAM15 supplementation (Supp. Table 2). EDL
225 muscles demonstrated an increase in the elasticity index, suggesting increased muscle stiffness in aged females
226 (Supp. Table 2). BAM15 supplementation did not affect muscle resilience to fatigue in female EDL muscles
227 (Figure 2C). Further, BAM15 did not affect contractility in female SOL muscles, with lower absolute and specific
228 force-frequency relationships, and reduced resilience to fatigue with lower force production during the recovery
229 period observed (Figure 2D). The rate of force development was lower in aged females (Supp. Table. 2), with no
230 additional significant findings found for SOL twitch characteristics.

231

232 **BAM15 supplementation protects mitochondrial respiration in aged male mice**

233 In permeabilised GTN muscle fibre bundles from males, BAM15 supplementation increased ADP-dependent
234 respiration, but did not alter ADP sensitivity (Figure 3A). Apparent Km data suggest that the mitochondrial
235 'fitness' to respond to ADP as a substrate is near completely diminished in aged muscles (~98% loss), due to
236 enzyme active sites reaching saturation at low ADP concentrations (Fig. 3B). BAM15 supplementation protected
237 against loss of Complex I leak respiration, suggestive of mild-uncoupling (Figure 3B). Further, BAM15
238 supplementation protected against age-related declines in Complex I and Complex I+II substrate-dependent
239 respiration, and maximally uncoupled respiration in sarcopenic male muscles (Figure 3B). To investigate whether
240 changes in mitochondrial respiration were connected to changes in mitochondrial content or oxidative stress, we
241 measured mitochondrial ETC complex and citrate synthase protein content via immunoblotting. There were no
242 changes to the protein content of mitochondrial ETC complexes I-V nor citrate synthase in aged male muscles
243 (Figure 3C-D). However, BAM15 supplementation protected against elevated 4-HNE content (lipid-peroxidation
244 marker) in aged male muscles (Figure 3C-D).

245 BAM15 supplementation did not protect against the reduction of ADP-dependent respiration and near-
246 complete ablation of ADP sensitivity (~97% loss) in aged female muscles (Figure 4A). We did not observe a loss
247 in Complex I leak respiration in 26M CHOW group. Subsequently, there was no change in mitochondrial
248 uncoupling observed through BAM15 supplementation (Figure 4B). Aged female muscles had lower Complex I
249 and Complex I+II substrate-dependent respiration, and maximally uncoupled respiration compared to young
250 reference controls, with no effect observed from BAM15 supplementation. Interestingly, we demonstrated a
251 reduction in Complex I subunit NDUFB8 content in aged female muscles (~30%), with some potential
252 remodelling of Complex IV and Complex III content from BAM15 supplementation evident (Figure 4C-D). These
253 findings may account, in part, for the diminished mitochondrial respiration. There were no further changes to
254 Complex II and V, citrate synthase or 4-HNE content in aged females (Figure 4C-D).

255

256 **BAM15 supplementation does not alter skeletal muscle mitophagy in aged mice**

257 BAM15 administration has previously been suggested to alter mitophagy-related protein content in a mouse
258 model of sarcopenic-obesity [10]. Thus, we employed the use of MitoQC mice to directly test whether BAM15
259 alters skeletal muscle mitophagy. In aged male mice, we demonstrate there are no changes to mitolysosome
260 abundance from BAM15 supplementation (Fig. 5A-C). When normalised to fibre area, there was no effect of age
261 or BAM15 supplementation observed on skeletal muscle mitophagy (Fig. 5D) However, we did observe an
262 increase in mitolysosome size in male 26M muscles (~52%, Fig. 5E). This may indicate alterations in mitophagic
263 processes or machinery which drive the accumulation of larger mitolysosomes. In aged female mice we observed
264 an increase in mitolysosome abundance (~76%) which is not rescued by BAM15 supplementation (Fig. 6A-C).
265 This is compatible with normalised data, with unsignificant trends indicating an increase in mitolysosome per
266 fibre area (Fig. 6D). Consistent with aged male data, female 26M mice demonstrated an increase in mitolysosome
267 size (~49%, Fig. 6E).

268

269 **Discussion**

270 Here we provide the first evaluation of the mitochondrial uncoupler BAM15 as a sarcopenia drug candidate in
271 male and female mice. We demonstrate that BAM15 supplementation does not alter age-associated loss in muscle

272 mass, but prevents a decline in skeletal muscle contractility and mitochondrial respiration leading to functional
273 improvements in sarcopenic muscle. Protection against age-related muscle functional decline was evident in both
274 male and female EDL muscle force production. This was consistent with previous investigation by Dantas *et al.*
275 where BAM15 supplementation improved 4-limb grip strength in aged high fat diet-induced obese mice [10].
276 Functional protection from BAM15 supplementation in aged muscle may be explained, in part, by the preservation
277 of muscle bioenergetics, observed as improved mitochondrial respiration and the restoration of oxidative stress
278 marker 4-HNE. Oxidative stress has been shown to negatively impact excitation-contraction coupling through
279 impaired $Ca^{2+}$ sensitivity in aged mice and mice with global deletion of CuZn superoxide dismutase, that have
280 lower antioxidant activity [19]. This mechanism linking preserved muscle bioenergetics is likely to indirectly
281 involve protection against motor neuron degeneration, which is required for skeletal muscle dysfunction during
282 sarcopenia [20].

283 In aged male mice, BAM15 supplementation enhanced skeletal muscle resilience to fatigue. This is likely related
284 to improved mitochondrial function but is difficult to definitively pinpoint, given the complex nature of skeletal
285 muscle fatigue [21]. In aged females, BAM15 supplementation did not show changes to fatigue resilience. We
286 suggest this is due to female aged EDLs having a lower delta change in contractile force production due to age
287 when compared to males (~107mN lower in female and ~241mN lower in male aged controls compared to young
288 references). In EDL muscle, there seems to be a clear relationship between muscle force production and muscle
289 resilience to fatigue which may be a better indicator of overall muscle performance rather than fatigue
290 susceptibility. Age-related muscle decline has a greater burden in predominantly fast type II myo-fibre muscles
291 [1], which aligns with our data in the predominantly type IIB myo-fibre EDL muscle. Despite being susceptible
292 to age-related functional decline, SOL muscle contractility data did not follow the same protective theme arising
293 from BAM15 supplementation. There are examples of paradoxical mechanistic regulation between EDL and SOL
294 muscles, such as global myostatin knockout mice, which exhibit lower specific force production in EDL muscles,
295 while SOL muscles remain unaffected compared to wild-type controls [22]. A simpler explanation could be that
296 SOL (mixed myo-fibre type) muscle does not undergo mitochondrial decline to the same extent as the EDL,
297 (predominately type II myo-fibre type) during aging [23].

298 Previous work has demonstrated that the mitochondrial-targeted antioxidant mitoquinone mesylate (mitoQ)
299 did not maintain EDL muscle force production of aged mice [24], whilst the mitochondrial-targeting peptide SS-
300 31 (elamipretide) improved treadmill running performance and reduced fatigue susceptibility without changes to

301 force production in tibialis anterior muscles of aged mice [25,26]. Further, delivery of untargeted, mito-protective
302 compounds such as $NAD^+$ precursors [27, 28], oleuropein [29] and Urolithin A [30], demonstrate improved
303 functional capacity and/or muscle contractility in aged mice. BAM15 therefore joins a short list of mitochondrial
304 targeted compounds that can improve sarcopenic muscle function.

305 BAM15 supplementation preserved substrate-dependent phosphorylating respiration and maximally
306 uncoupled ETC-linked respiration without any changes in mitochondrial protein content in aged male mice. To
307 date, *in vivo* data has only shown that BAM15 supplemented in high-fat diet can increase mitochondrial content
308 and Complex II-IV activity in skeletal muscles of aged male mice [10]. In our hands, BAM15 supplementation
309 protected mitochondrial function, which is underscored by the attenuation of the oxidative stress marker, 4-HNE.
310 Tsuji *et al.* previously demonstrated that BAM15 improves survival in a mouse model of sepsis by protecting
311 kidneys from oxidative damage and lowering circulating mtDNA [31]. These findings are linked to the mild
312 uncoupling induced by BAM15, a targeted mechanism that has long been considered to reduce
313 oxidative/nitrosative stress and restore normative mitochondrial function [6,32]. This will also lead to lower ROS
314 production rate and protection against unchecked oxidative damage from reactions instigated by single electron
315 reductants that can react with free oxygen and lead to superoxide ($O_2^-$) formation [7]. Mild uncoupling induced
316 by BAM15 supplementation is therefore a promising anti-oxidant strategy to protect tissues from oxidative
317 damage and mitochondrial respiration.

318 Skeletal muscle from aged female mice demonstrated a different mitochondrial phenotype compared to males.
319 Like male counterparts, aged female mice had lower substrate-dependent phosphorylating and maximally-
320 uncoupled respiration. However, this was not underlined by changes in oxidative stress or mitochondrial content
321 markers, rather a ~30% loss of Complex I subunit NDUFB8 protein content. To the best of our knowledge, this
322 finding has not previously been observed. Recently, data from Moreira-Pais *et al.* suggested that the age-dependent
323 remodelling of the skeletal muscle mitochondrial proteome is more pronounced in females due to reduced serum
324 17β-estradiol levels and the mitochondrial pool of estrogen receptor alpha (ERα) [33]. These authors also
325 identified downregulation of Complex 1 subunit NDUFV2 from proteomic analysis [33], suggesting some overlap
326 between our findings. In aged female muscles we also observe higher rates of mitophagy, with no changes in
327 males.

328 Loss of ADP sensitivity is considered to be a key representation of the metabolic decline in aged human
329 skeletal muscle, which is a reversible bioenergetic target of resistance training [34]. However, our data show that

330 aged mouse muscles show complete loss of the metabolic fitness to respond to ADP as a substrate, with near-
331 complete saturation observed after 100 μM of ADP, regardless if supplemented with BAM15. Pharaoh *et al.* found
332 that there is also an increase in the amount of ADP required to reduce $H_2O_2$ production in aged mouse muscle
333 [35]. This was underlined by lower content of proteins involved in the transport of ADP into mitochondria,
334 ADP/ATP translocase 2 (ANT2) and the voltage-dependent anion channel (VDAC) [35]. Additionally, there was
335 no change in mitophagy levels from BAM15 supplementation in aged muscles. However, there was some
336 indication of altered mitophagic processes, with larger mitolysosomes identified in both aged male and female
337 muscles [36, 37]. Collectively, these data suggest that there is likely a nuanced redox-dependent mechanism that
338 regulates the relative loss of metabolic fitness to respond to ADP as a substrate and ADP transporter abundance,
339 which in turn can alter mitophagy in aged muscles.

340 In summary, BAM15 supplementation preserved EDL contractile function and mitochondrial respiration
341 without altering age-related loss in muscle mass. Future investigations should explore both earlier and extended
342 treatment durations for BAM15 supplementation, given that age-related muscle decline is typically considered to
343 commence at ~18 months of age in mice. Our findings suggest that maintaining mitochondrial efficiency and
344 redox balance may mitigate functional decline in sarcopenic muscle. Together, our data support mitochondrial
345 uncoupling as a promising therapeutic strategy to preserve muscle function during the progression of sarcopenia.

346

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351

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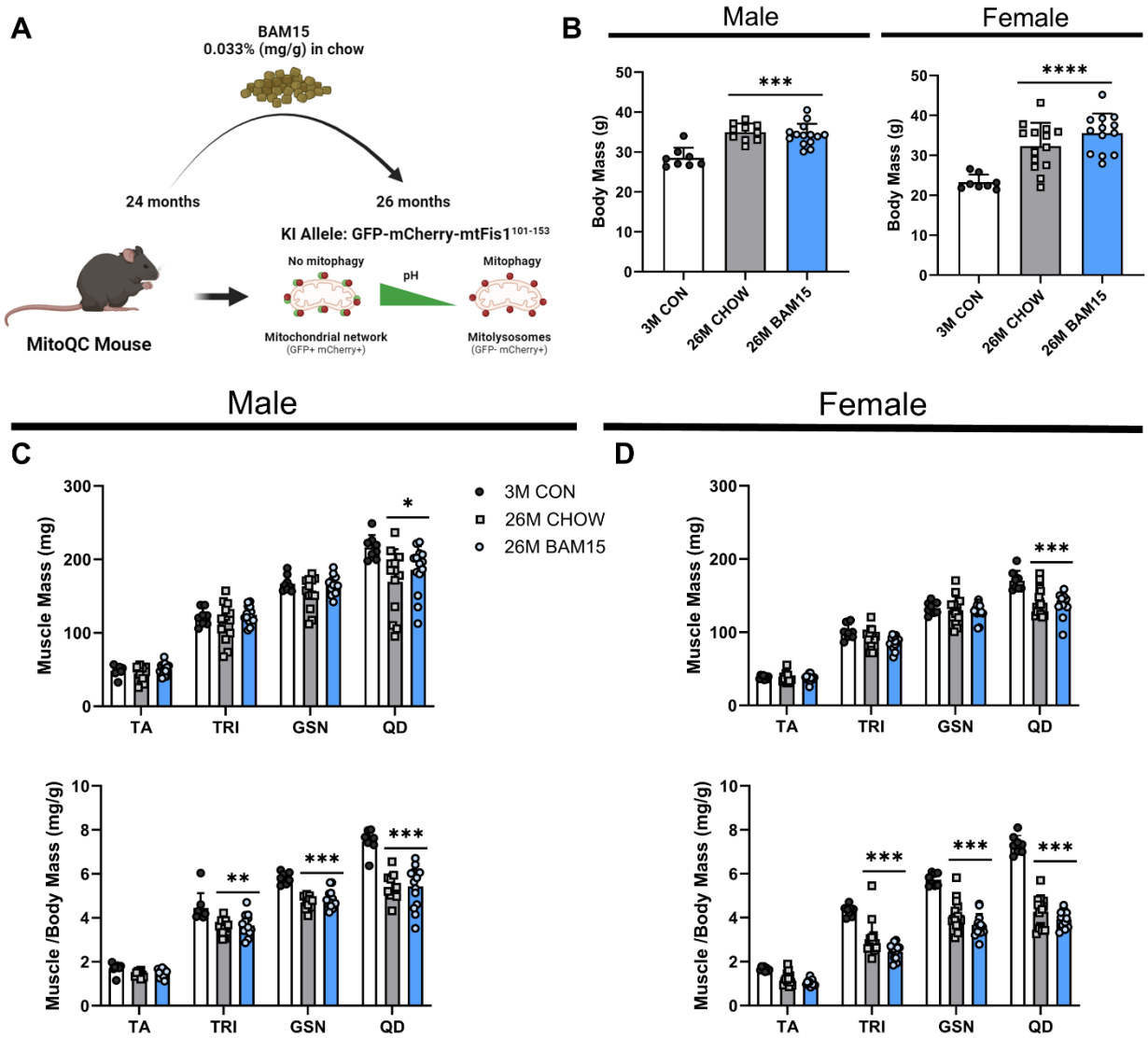
462 **Figures:**




463

464 **Figure 1: Body mass and muscle mass are unchanged from BAM15 supplementation in aged sarcopenic**
465 **mice.** A) Infographic of the mouse model and experimental design. B) Endpoint body mass, C) skeletal muscle
466 mass (TA: tibialis anterior; TRI: triceps; GSN: gastrocnemius; QD: quadriceps) and D) skeletal muscle to body
467 mass ratios shown. Data is presented as mean ± SD. n=7-8 per group for 3M CON; n=13-14 per group for 26M
468 males and females. *$p$,0.05; **$p$<0.01; ***$p$<0.001 vs 3M CON.

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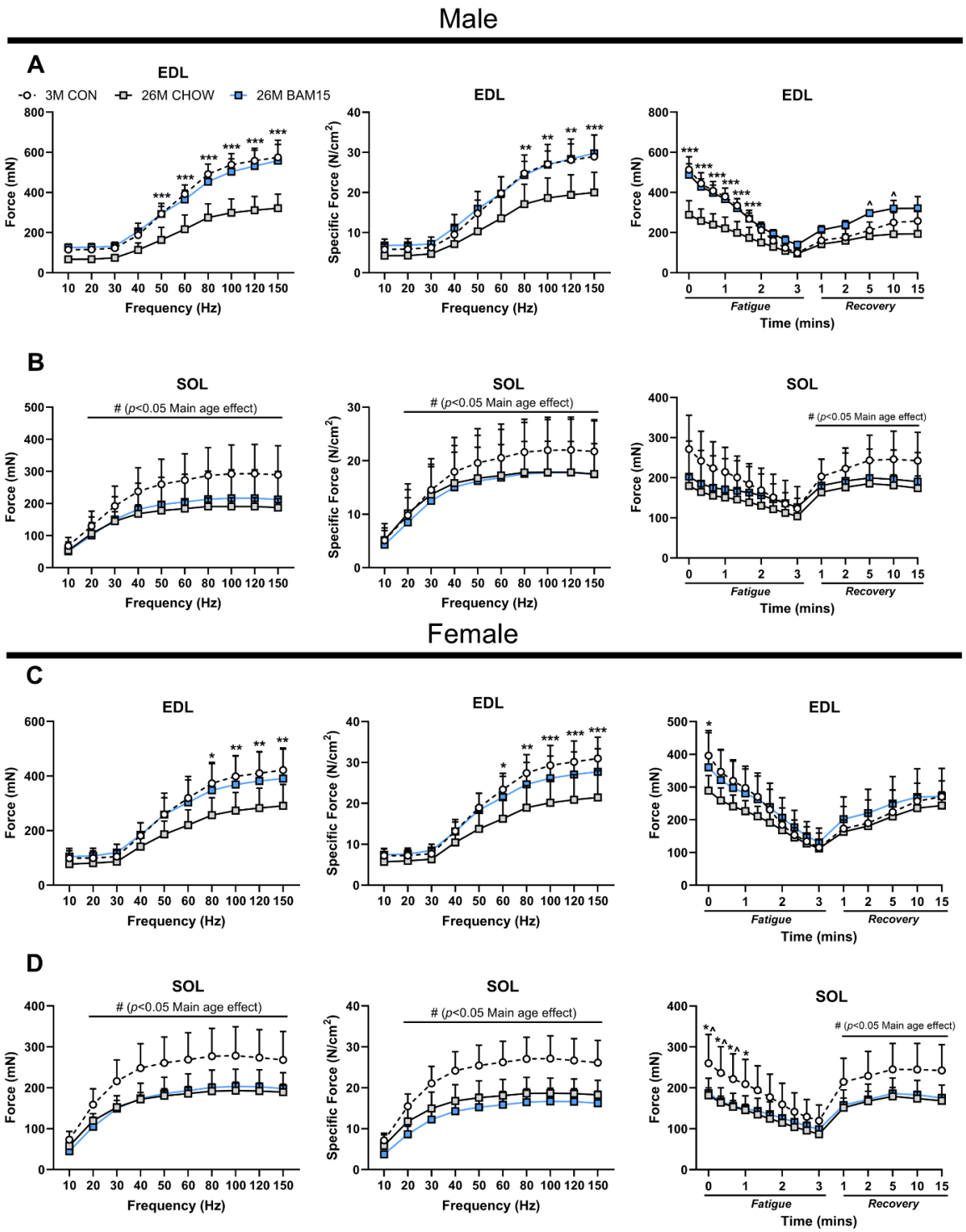


471

472 **Figure 2: BAM15 supplementation improves EDL muscle contractile function in sarcopenic male and**
473 **female mice.** Ex vivo contractile function assessment was performed in extensor digitorum longus (EDL) and
474 soleus (SOL) muscles. Male A) EDL and B) SOL muscles are analysed with force-frequency relationships
475 showing absolute and specific force (normalised to physiological cross-sectional area) production across a range
476 of frequencies in the EDL. Muscle resilience was tested with absolute force produced during the fatigue stimulus

and recovery period shown. This contractile testing battery was repeated in female C) EDL and D) SOL muscles. Data is presented as mean ± SD. n=8 for 3M CON males and n=5-6 per group for 26M old males. n=8-9 per group for females. *$p<0.05$; **$p<0.01$; ***$p<0.001$ 26M CON vs 3M CON. ^ $p<0.05$ 26M BAM15 vs 3M CON.

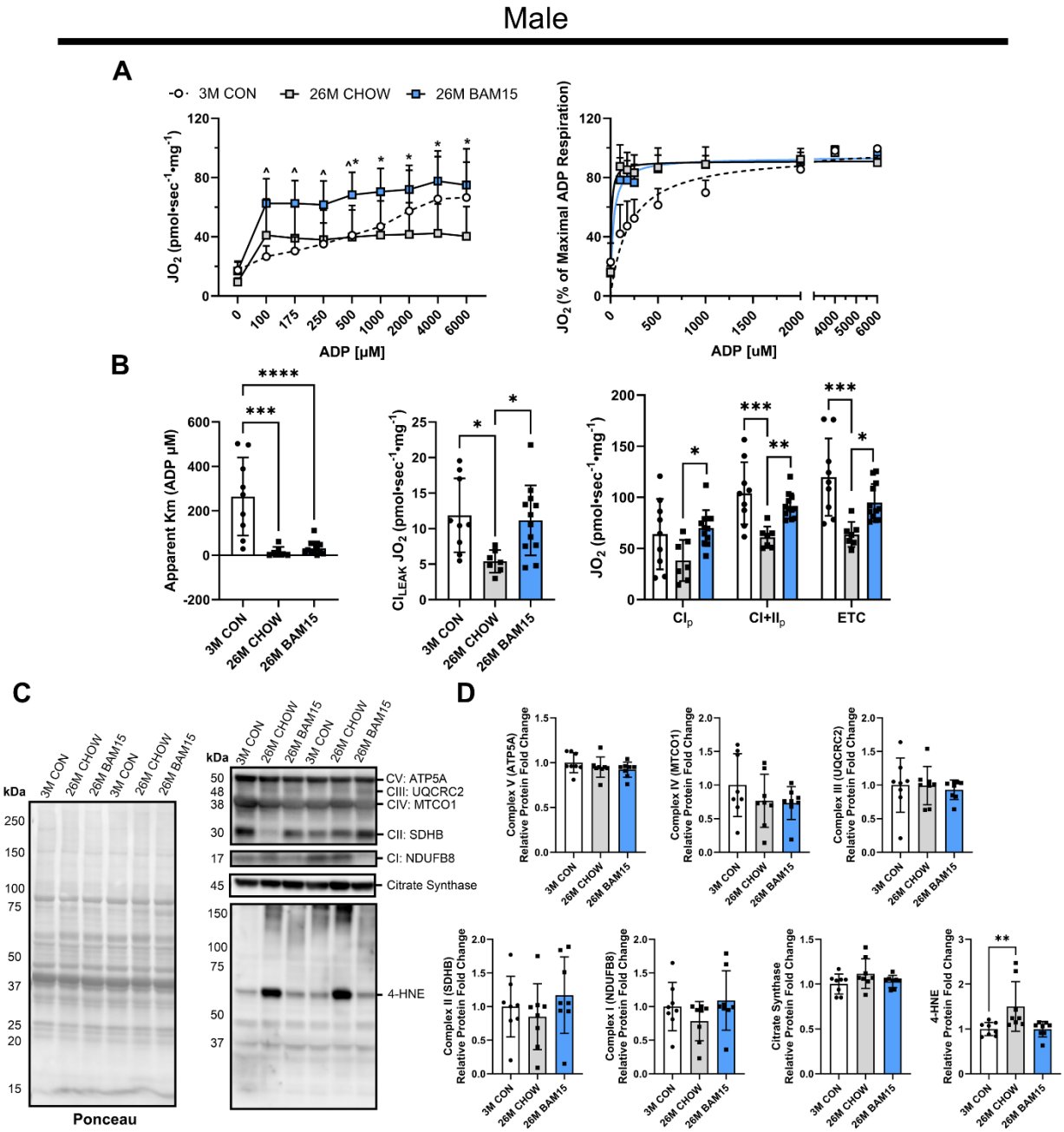


**Figure 3: BAM15 supplementation preserves skeletal muscle mitochondrial respiration but does not impact content abundance in sarcopenic male mice.** Using permeabilized fibre bundles from male gastrocnemius muscles mitochondrial respiration was assessed via high resolution respirometry. A) Respiration is stimulated via an ADP titration in the presence of pyruvate and malate with standard and transformed data (via the Michaelis–Menten kinetics equation) presented. B) Apparent Km which represents the required concentration of ADP required to reach 50% of the maximal ADP-dependent respiration, Complex 1 Leak respiration, phosphorylating respiration ($CI_p$) in the presence of Complex I substrate (glutamate) and Complex I+II substrates (glutamate and succinate – $CI+II_p$) and CCCP-induced maximal uncoupled respiration are shown. Protein abundance was

measured via immunoblotting with OXPHOS subunits Complex V (ATP5A), Complex IV (MTCO1), Complex III (UQCRC2), Complex II (SDHB) and Complex I (NDUFB8), alongside Citrate Synthase and 4-Hydroxynonenal (4-HNE) probed. C) Representative images for antibodies and Ponceau for protein loading are shown and D) data quantified relative to 3M CON. Data is presented as mean ± SD. n=7-12 per group for mitochondrial respiration analyses; n= 7-8 per group for immunoblotting analyses. *$p$,0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001 26M CON vs 3M CON. ^ $p$<0.05 26M BAM15 vs 3M CON.

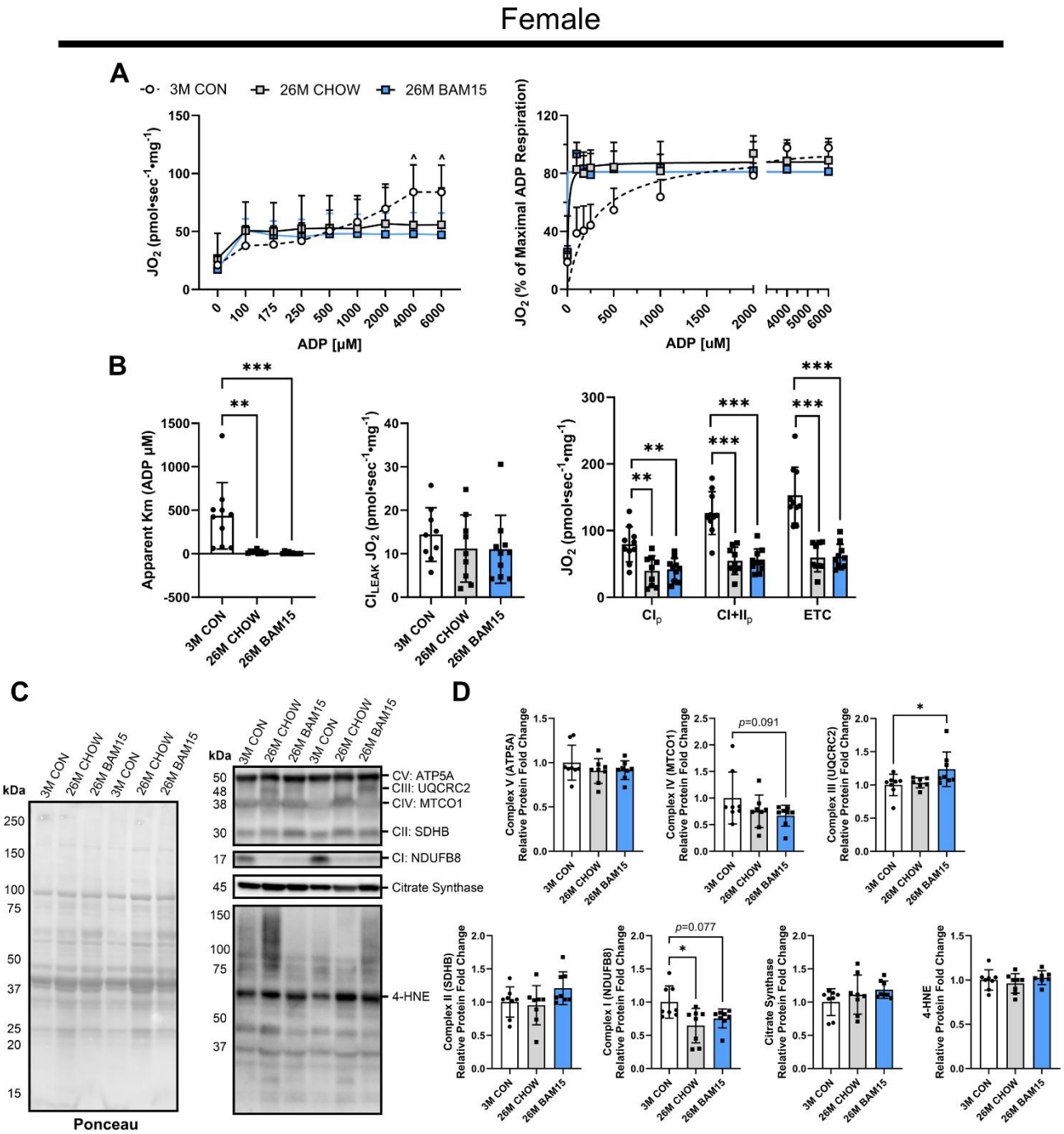


**Figure 4: BAM15 supplementation does not alter skeletal muscle mitochondrial respiration or content abundance in sarcopenic female mice.** Using permeabilized fibre bundles from male gastrocnemius muscles mitochondrial respiration was assessed via high resolution respirometry. A) Respiration is stimulated via an ADP titration in the presence of pyruvate and malate with standard and transformed data (via the Michaelis–Menten kinetics equation) presented. B) Apparent Km which represents the required concentration of ADP required to reach 50% of the maximal ADP-dependent respiration, Complex 1 Leak respiration, phosphorylating respiration

($CI_P$) in the presence of Complex I substrate (glutamate) and Complex I+II substrates (glutamate and succinate – $CI+II_P$) and CCCP-induced maximal uncoupled respiration are shown. Protein abundance was measured via immunoblotting with OXPHOS subunits Complex V (ATP5A), Complex IV (MTCO1), Complex III (UQCRC2), Complex II (SDHB) and Complex I (NDUFB8), alongside Citrate Synthase and 4-Hydroxynonenal (4-HNE) probed. C) Representative images for antibodies and Ponceau for protein loading are shown and D) data quantified relative to 3M CON. Data is presented as mean ± SD. n=9-10 per group for mitochondrial respiration analyses; n= 7-8 per group for immunoblotting analyses. *$p$,0.05; **$p$<0.01; ***$p$<0.001; ****$p$<0.0001 26M CON vs 3M CON. ^ $p$<0.05 26M BAM15 vs 3M CON.

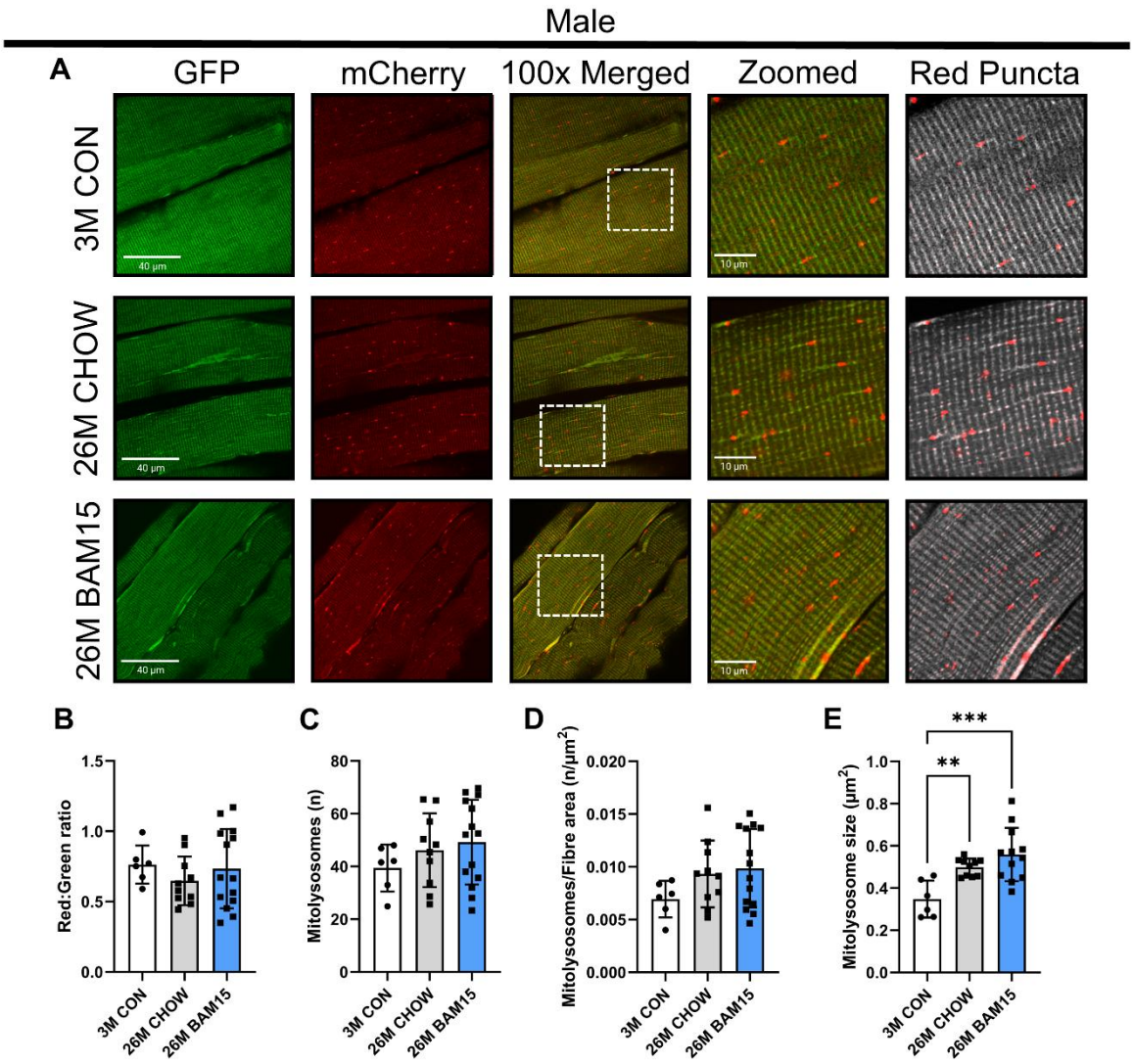


**Figure 5: BAM15 supplementation does not alter skeletal muscle mitophagy status in sarcopenic male mice.** Fixed quadriceps muscles were embedded in OCT and longitudinally sectioned for mitophagy quantitation via measurement of MitoQC reporter fluorophores GFP and mCherry using confocal microscopy. A) Representative images are displayed, alongside B) mean intensity ratio of the red and green channels, C) total number of mitolysosomes, D) mitolysosomes per fibre area and E) mitolysosome size reported. Data is presented as mean ± SD. 15-17 fibres analysed per section. n=6 for 3M CON; n= 10-14 per group for 26M males. *$p$,0.05; **$p$<0.01; ***$p$<0.001; 26M CON vs 3M CON.

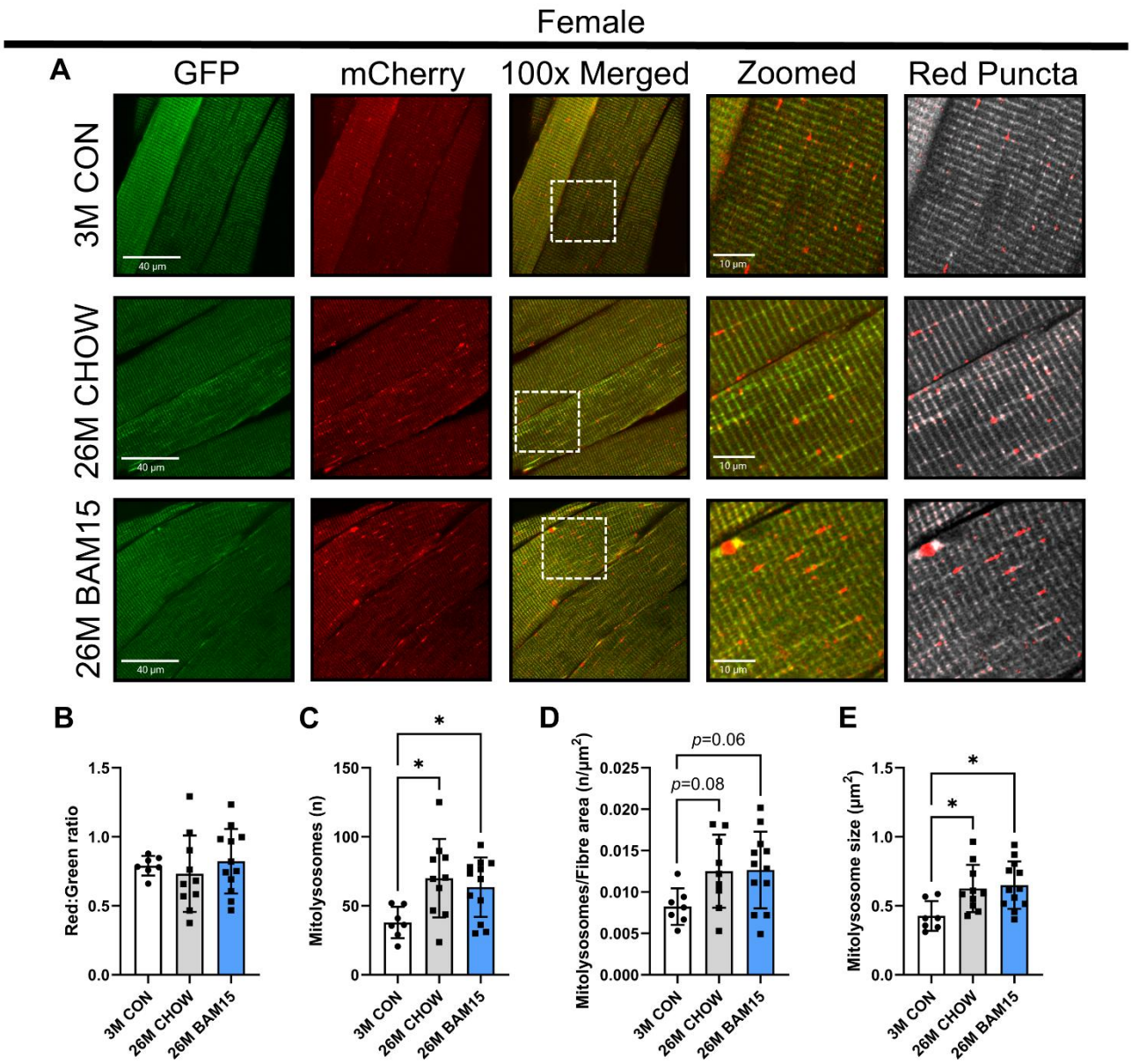


**Figure 6: BAM15 supplementation does not alter skeletal muscle mitophagy status in sarcopenic female mice.** Fixed quadriceps muscles were embedded in OCT and longitudinally sectioned for mitophagy quantitation via measurement of MitoQC reporter fluorophores GFP and mCherry using confocal microscopy. A) Representative images are displayed, alongside B) mean intensity ratio of the red and green channels, C) total number of mitolysosomes, D) mitolysosomes per fibre area and E) mitolysosome size reported. Data is presented as mean ± SD. 15-17 fibres analysed per section. n=7 for 3M CON; n= 10-12 per group for 26M females. *$p$,0.05; **$p$<0.01; ***$p$<0.001; 26M CON vs 3M CON.