

# Is the Ergogenicity of Caffeine Affected by Increasing Age? The Direct Effect of a Physiological Concentration of Caffeine on the Power Output of Maximally Stimulated EDL and Diaphragm Muscle Isolated From the Mouse

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1 **Is the Ergogenicity of Caffeine Affected by Increasing Age? The Direct Effect of a Physiological**  
2 **Concentration of Caffeine on the Power Output of Maximally Stimulated EDL and Diaphragm**  
3 **Muscle Isolated From the Mouse**

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28 **Abstract**

29 *Introduction:* Caffeine is a well-established performance enhancing nutritional supplement in a young  
30 healthy population, however far less is known about how its ergogenicity is affected by increasing  
31 age. A recent review has highlighted the value of studies examining the direct effect of caffeine on  
32 isolated skeletal muscle contractility, but the present work is the first to assess the direct effect of  
33 70 $\mu$ M caffeine (physiological maximum) on the maximal power output of isolated mammalian  
34 muscle from an age range representing developmental to early ageing. *Method:* Female CD1 mice  
35 were aged to 3, 10, 30 and 50 weeks (n = 20 in each case) and either whole EDL or a section of the  
36 diaphragm was isolated and maximal power output determined using the work loop technique. Once  
37 contractile performance was maximised, each muscle preparation was treated with 70 $\mu$ M caffeine and  
38 its contractile performance was measured for a further 60 minutes. *Results:* In both mouse EDL and  
39 diaphragm 70 $\mu$ M caffeine treatment resulted in a significant increase in maximal muscle power  
40 output that was greatest at 10 or 30 weeks (up to 5% & 6% improvement respectively). This  
41 potentiation of maximal muscle power output was significantly lower at the early ageing time point,  
42 50 weeks (up to 3% & 2% improvement respectively), and in mice in the developmental stage, at 3  
43 weeks of age (up to 1% & 2% improvement respectively). *Conclusion:* Uniquely, the present findings  
44 indicate a reduced age specific sensitivity to the performance enhancing effect of caffeine in  
45 developmental and aged mice which is likely to be attributed to age related muscle growth and  
46 degradation, respectively. Importantly, the findings indicate that caffeine may still provide a  
47 substantial ergogenic aid in older populations which could prove important for improving functional  
48 capacity in tasks of daily living.

49 **Key Words:** Dynapenia, Ergogenic Aid, Force, Sarcopenia, Skeletal Muscle, Work Loop

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## 53 **Introduction**

54 The ergogenic benefit of caffeine to promote performance enhancing effects has been extensively  
55 studied and it is well recognised that caffeine induces increases in endurance, strength and power  
56 performance in young healthy participants [see reviews 6, 16, 23]. Given that large amounts of  
57 caffeine are consumed by a broad age range of people in western societies, there is surprisingly little  
58 evidence examining how the ergogenicity of this drug is affected by age. However, with an  
59 increasingly aged population whose decline in skeletal muscle function affects performance in  
60 everyday tasks [60], research is warranted to examine the age specific sensitivity of skeletal muscle to  
61 the direct performance enhancing effects of caffeine.

62 Norager et al. [41] was one of the first studies to examine the effect of acute caffeine supplementation  
63 on exercise performance in an elderly population. The study reported a significant increase in cycling  
64 endurance (25%), arm flexion endurance (54%) and associated reduction in RPE in men and women  
65 aged over 70 following 6mg/kg caffeine consumption. No significant differences were found in  
66 muscle strength, walking speed, or reaction time. Interestingly, Norager et al, [41] reported that the  
67 increase in arm flexion endurance was markedly higher than that seen in an earlier study on younger  
68 individuals. This suggests that caffeine may prove to be more ergogenic in an older adult population.  
69 In support of this notion, Swift and Tiplady [52] further concluded that the elderly may be more  
70 sensitive to the effects of caffeine at the psychomotor level with this population showing greater  
71 improvements in attention and choice reaction time compared to younger participants. Duncan et al,  
72 [20] demonstrated that low dose (3mg/kg) caffeine improved six minute walk distance, 8 foot up and  
73 go time, number of arm curl reps completed in 30 seconds and manual dexterity in participants aged  
74 66 years. Subsequently Duncan et al, [20] concluded that caffeine could be used as an effective  
75 nutritional supplement for improving functional performance in the elderly. In agreement, Momsen et  
76 al, [39] demonstrated a significant increase in maximal walking distance (20%) and maximal  
77 isometric knee extension strength (9.8%) following 6 mg/kg caffeine supplementation in 68 year old  
78 patients with moderate intermittent claudication. Conversely, evidence suggests that a caffeine  
79 induced increase in muscular strength of elderly participants is not always reported [54]. This

80 relatively small quantity of research and the ambiguity in findings warrant further more controlled  
81 research examining the relationship between the performance enhancing effects of caffeine and  
82 increasing age.

83 A recent review by Tallis et al, [53] has highlighted the value of isolated mammalian muscle studies  
84 for examining the direct performance enhancing effect of caffeine. As the mechanism of enhanced  
85 performance has in part been attributed to the direct action of caffeine to promote an increase in  
86 muscle function [37], such in vitro studies allow the direct assessment of caffeine on skeletal muscle  
87 without interference from other central and physiological processes. More importantly digestion and  
88 distribution, habituation, withdrawal effects, side effects of high dose caffeine consumption and  
89 motivation to complete repeated maximal exercise, that are factors difficult to control in human  
90 studies, are not prevalent in isolated muscle work. Such aspects have been suggested to result in an  
91 individual caffeine response subsequently resulting in a number of equivocal findings in the human  
92 literature [53]. The same limitations affect the relatively small amount of literature examining the  
93 effect of caffeine on performance in older human participants and subsequently warrant the use of  
94 isolated muscle studies to get a clearer indication of the effectiveness of this drug in such populations.

95 Previous work, using isolated muscles from young healthy rodents, has demonstrated that high (mM),  
96 and more recently, physiologically relevant (50-70 $\mu$ M) concentrations of caffeine can cause a  
97 significant fibre type specific improvement in the maximal force and power producing properties of  
98 skeletal muscle [3, 28, 29, 55, 56]. Mechanistically this has been attributed to the action of caffeine as  
99 an adenosine receptor antagonist acting specifically at the A1 receptors on the skeletal muscle  
100 membrane and/or its ability to bind to ryanodine receptors (RYR) of the sarcoplasmic reticulum (SR)  
101 [14, 21, 46]. The net effect is an improvement in excitation contraction coupling promoting greater  
102 SR Ca<sup>2+</sup> release, and as a consequence, a more forceful contraction [23, 33, 55]. An increase in age is  
103 associated with a reduction in the efficacy of the excitation contraction coupling process and altered  
104 Ca<sup>2+</sup> handling properties as a mechanism for the age related loss of muscle function [17, 40]. These  
105 age related changes in E-C coupling may significantly reduce the skeletal muscle response to caffeine

106 treatment and mechanistically would appear to contradict the increased caffeine sensitivity proposed  
107 by Norager et al, [41].

108 The present study builds on our previous body of work (reviewed in [53]) and is the first to assess the  
109 direct effects of a physiologically relevant concentration of caffeine (70 $\mu$ M, maximal for human  
110 consumption; [21]) on mammalian skeletal muscle performance in a large age range of individuals.  
111 Specifically our aim was to measure the maximal power output of isolated mouse EDL  
112 (predominantly fast-twitch) and diaphragm (mixed muscle fibre type) skeletal muscles over an age  
113 range from ‘developmental’ (3 weeks old) to ‘early aged’ (50 weeks old). Previous evidence has  
114 demonstrated that 50 week old CD1 mice show a substantial age related decline in skeletal muscle  
115 mechanical performance [57] and were subsequently deemed appropriate for use in the context of this  
116 study. Moreover, the present work is the first (*in vivo or in vitro*) study to examine the ergogenic  
117 effect of caffeine over a broad range of ages, rather than just examining older adults or younger adults  
118 as in previous human work [6, 20, 23, 41, 56]. By using the work loop technique to examine different  
119 muscles, the present study will be able to establish fibre type specific effects of caffeine on the  
120 mechanical properties of muscle in relation to age using a method that better simulates real life  
121 muscle function than more commonly used *in vitro* tests [27, 30, 31, 32]. In addition, the ergogenic  
122 effect of caffeine on exercise performance has not been studied in children and adolescents despite the  
123 high consumption of caffeinated products in this population [5]. The present study will offer an  
124 important insight into the direct effects of caffeine in juvenile and elderly populations.

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131 **Methods**

132 *Animals*

133 Female white mice (strain CD1 mice, Charles River, UK) were bred and kept in house at Coventry  
134 University. This study was approved by the Coventry University ethics committee. The assessment of  
135 the age related direct effect of caffeine was conducted on mice aged 3, 10, 30 and 50 weeks old (n=20  
136 in each case). From birth animals were kept in groups of 8 without access to running wheels. The  
137 rationale for the use of animals at these age groups to examine the ageing effect on mechanical  
138 performance of skeletal muscle is given in our previous work [57] but can be summarised as follows  
139 for both EDL and diaphragm: At 3 weeks of age mice are weaned and muscle performance is low, at  
140 10 weeks muscle performance peaks, at 30 weeks the onset of ageing begins and by 50 weeks there is  
141 a substantial age related reduction in the maximal force and power generating capacity of various  
142 skeletal muscles.

143 *Dissection*

144 Mice were weighed to the nearest 0.1g on an electronic balance (body mass (g): 3 weeks =  $15.8 \pm$   
145  $1.2$ ; 10 weeks =  $31.9 \pm 0.8$ ; 30 weeks =  $41.6 \pm 0.8$ ; 50 weeks  $58.2 \pm 5.4$ ; mean  $\pm$  SE, n=20 in each age  
146 group). Mice were then killed by cervical dislocation in accordance with the British Home Office  
147 Animals (Scientific Procedures) Act 1986, Schedule 1. Either a ventral section of the costal  
148 diaphragm or an EDL muscle was dissected from each mouse in cooled (4-6°C) oxygenated (95% O<sub>2</sub>;  
149 5% CO<sub>2</sub>) Krebs-Henseleit solution of composition (mM) NaCl 118; KCl 4.75; MgSO<sub>4</sub> 1.18; NaHCO<sub>3</sub>  
150 24.8; KH<sub>2</sub>PO<sub>4</sub> 1.18; glucose 10; CaCl<sub>2</sub> 2.54; pH 7.55 at room temperature prior to oxygenation. As a  
151 method to prevent tendon slippage during muscle force production, aluminium foil T-clips were  
152 wrapped around each tendon of the EDL. The central section of the left hand half of the diaphragm  
153 muscle was prepared in a similar fashion, however at one end the two ribs that anchored the muscle  
154 were left intact.

155 *Experimental Set-Up*

156 Using either the aluminium foil T-clips or bone, each muscle preparation was connected via crocodile  
157 clips to a force transducer at one end (UF1, Pioden Controls Ltd, Kent, UK) and a motor at the  
158 opposing end (V201, Ling Dynamic Systems, Hertfordshire, UK). Position of the motor arm was  
159 detected via a Linear Variable Displacement Transformer (DFG5.0, Solartron Metrology, Sussex,  
160 UK). The muscle was maintained in circulated oxygenated Krebs-Henseleit solution at a constant  
161 temperature of 37°C. The muscle was electrically stimulated to produce force via parallel platinum  
162 electrodes inside the muscle chamber. These electrodes were not directly in contact with the muscle or  
163 the nerve branch but caused activation by stimulating the surrounding fluid. Muscle stimulation and  
164 length change parameters were controlled using custom written software (Testpoint, CEC,  
165 Massachusetts, USA) via a D/A board (KPCI3108, Keithley Instruments, Ohio, USA) on a desktop  
166 PC. Each muscle preparation went through a series of isometric muscle tests followed by assessments  
167 of work loop power.

#### 168 *Isometric Testing*

169 Initially twitch force was assessed by electrically stimulating the muscle whilst held at a constant  
170 length. The muscle length and stimulation amplitude (14-18V for EDL; 10-16V for diaphragm) were  
171 optimised to produce maximal twitch force. The muscle length that corresponded with maximal  
172 twitch force was measured using an eyepiece graticule and defined as  $L_0$ . Similarly to James *et al*  
173 [27], mean muscle fibre length was calculated as 75% of  $L_0$  for EDL muscle. As no such estimate of  
174 fibre length exists for diaphragm the direct measurement taken was used as  $L_0$  [57]. Each muscle was  
175 then subjected to a 250ms burst of electrical stimulation in order to produce a tetanus response.  
176 Stimulation frequency was optimised in order to produce maximal tetanus force (usually 200Hz for  
177 EDL and 140Hz for diaphragm; this was not affected by age). A 5 minute rest period was imposed  
178 between each tetanus in order to allow the muscle sufficient recovery between stimulations.

#### 179 *The Work Loop Technique*

180 The work loop technique was employed as a method of providing a closer representation of *in vivo*  
181 skeletal muscle performance [31]. Similarly to *in vivo* muscle function, this method considers that



182 muscle cannot shorten indefinitely and must go through a period of re-lengthening before subsequent  
183 contraction. The work loop technique employs cyclical length changes using waveforms and  
184 stimulation parameters that more closely approximate those used *in vivo* to assess the ability of the  
185 muscle to produce dynamic power [28, 30, 31, 32].

186 Essentially the approach used for EDL and diaphragm was the same as that used in our previous study  
187 on ageing [57]. The muscle was held at the previously determined  $L_0$  and the stimulation amplitude  
188 and stimulation frequency parameters that yielded maximal tetanic force were employed. Each muscle  
189 was subjected to four sinusoidal length change cycles per set at a total symmetrical strain of 0.10, as  
190 this strain has been used previously to elicit maximal power output in these muscles [27, 55, 57]. As  
191 such, the muscle lengthened by 5% from  $L_0$  followed by a shortening to 5% shorter than  $L_0$  before  
192 returning back to  $L_0$ . A cycle frequency of 10Hz and 7Hz was used for EDL and diaphragm muscle  
193 respectively. 10Hz represents the cycle frequency that has previously been shown to elicit maximal  
194 power output in EDL [27]. 7Hz represents the cycle frequency that elicited maximal power output in  
195 diaphragm in preliminary work by the authors and is similar to previous findings [4]. The magnitude  
196 and frequency of length changes and electrical stimulation were controlled via the Testpoint software.  
197 Data were sampled at a rate of 10 kHz and then a work loop was formed, by plotting force against  
198 length, the area of which represents the net work done by the muscle during a single length change  
199 cycle [31]. Stimulus burst duration (stimulation primarily through shortening) was altered until  
200 maximal net power output was achieved.

201 Usually a 49 ms burst duration was used for EDL which is in keeping with that previously used at  
202 10Hz cycle frequency [27, 57]. The burst duration commonly used to elicit maximum power output in  
203 diaphragm muscle was 55 ms, similar to our previous work [57]. On occasions the burst duration had  
204 to be increased or decreased to adjust the number of stimuli given. This was determined by examining  
205 the work loop power output and by interpretation of the work loop shapes. e.g. if the muscle is too  
206 active during re-lengthening it will significantly distort the shape of the loop and reduce muscle power  
207 output by increasing the resistance of the muscle to stretch. A stimulation phase shift of -2 ms and -5  
208 ms were used for EDL and diaphragm respectively, as they corresponded with maximal power output,

209 in agreement with our previous work [57]. So for EDL this stimulation phase shift value dictates that  
210 stimulation of the muscle starts 2 ms prior to the muscle reaching maximal length.

#### 211 *The Effect of 70 $\mu$ M Caffeine Treatment*

212 Each muscle was subjected to a set of 4 work loop cycles every ten minutes over a 130 minute  
213 duration [28, 55, 57]. Three measurements of the muscles maximal power output were made in  
214 standard Krebs-Henseleit solution and this formed the initial control baseline measurement. Following  
215 this the circulating fluid was changed to Krebs containing 70 $\mu$ M caffeine and a further 6  
216 measurements of maximal power output were made. The assessment concluded with a washout period  
217 where the circulating fluid was replaced with standard Krebs and a further 4 measurements of  
218 maximal power were taken, the latter three of which were used to represent the washout control.

#### 219 *Muscle Mass Measurements and Dimension Calculations*

220 At the end of the experiment the muscle was removed from the clips and the tendons were removed  
221 leaving the whole muscle intact. The muscle was then blotted on absorbent paper to remove excess  
222 fluid. The muscle was then placed on an electronic balance (Mettler Toledo B204-S, Zurich,  
223 Switzerland) to determine the wet muscle mass to the nearest 0.0001g. Mean muscle cross-sectional  
224 area was calculated from mean fibre length (muscle length in the case of diaphragm), muscle mass  
225 and an assumed muscle density of 1060 kg m<sup>-3</sup> [38]. Isometric stress was calculated as maximal  
226 tetanic force divided by mean muscle cross-sectional area. Muscle power output was normalised to  
227 muscle mass to express power as W.kg<sup>-1</sup>.

#### 228 *Statistical Analysis of the Data*

229 In control conditions muscle power output will decrease over time due to the gradual development of  
230 an anoxic core [8]. Over the 130 minute duration of the protocol used in the present study, muscle  
231 power output had decreased to 92.5 $\pm$ 0.61% of initial power output. In order to avoid the given  
232 deterioration in muscle power output masking the effects of the caffeine treatment, a 1<sup>st</sup> order  
233 regression equation was calculated using the pre-treatment control data and post treatment washout

234 control data to identify the linear relationship between muscle power output and time. This regression  
235 equation was then used to determine theoretical control muscle power output for each time point  
236 during caffeine treatment [28, 55].

237 Initially for each treatment group, pre-treatment controls were compared directly against post  
238 treatment washout controls using a paired t-test. There was no significant difference between these  
239 measurements (Paired T-test  $p > 0.7$  in each individual case) therefore these results were pooled to  
240 form controls. Thereafter, it was assumed that any subsequent change in muscle power output was  
241 solely the effect of the given caffeine treatment. Consequently controls were compared directly  
242 against caffeine treatment using a further paired T-test for each treatment group. To establish whether  
243 a difference in the effect of caffeine occurred between ages a single factor ANOVA was conducted on  
244 the caffeine treatment data, after normalisation as a percentage of the theoretical control, for each of  
245 EDL and diaphragm muscle respectively. When the ANOVA indicated a significant difference  
246 between age groups, Tukey post hoc tests were used to identify where these differences occurred. An  
247 independent samples t-test was conducted to compare the caffeine treatment results for EDL and  
248 diaphragm of the same age group in order to identify whether the ergogenic benefit was greater in a  
249 particular muscle at each age.

250 As the caffeine response was not uniform within each treatment group and EDL muscle mass was  
251 significantly affected by age (ANOVA  $p < 0.001$ ), we considered it valuable to determine if the  
252 caffeine effect was affected by muscle mass. This relationship was determined by using Pearson's two  
253 tailed correlation analysis for these data for each treatment group.

254 Results were interpreted as significant when  $p < 0.05$ . Values are displayed as mean  $\pm$  standard error.

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260 **Results**

261 *The Effect of 70 $\mu$ M Caffeine Treatment on the Maximal Power Output of Mouse EDL*

262 Treatment of mouse EDL with 70 $\mu$ M caffeine resulted in a significant increase in power output by up  
263 to 1%, 4%, 5% and 3% for 3, 10, 30 and 50 week old mice respectively (Fig 1; paired t-test  $p < 0.001$   
264 between control and caffeine power output in all cases). There was a significant effect of age on the  
265 caffeine induced improvement in power output (ANOVA  $p < 0.001$ ). The caffeine induced increase in  
266 muscle PO was the highest at 30 weeks and was significantly greater than the response at 3 and 50  
267 weeks (Fig 1; Tukey  $p < 0.001$  in both cases) and had a tendency to be greater than that at 10 weeks  
268 (Fig 1; Tukey  $p = 0.079$ ). The ergogenic benefit at 3 weeks was significantly lower than at all other  
269 ages (Fig 1; Tukey  $p < 0.005$  in all cases). The increase in muscle PO at 10 weeks did not prove to be  
270 significantly different to that at 50 weeks (Fig 1; Tukey  $p = 0.733$ ).

271 *The Effect of 70 $\mu$ M Caffeine Treatment on the Maximal Power Output of Mouse Diaphragm*

272 Treatment of mouse diaphragm with 70 $\mu$ M caffeine resulted in a significant increase in power output  
273 by up to 2%, 6%, 4%, and 2% for 3, 10, 30 and 50 week old mice respectively (Fig 2; paired t-test  
274  $p < 0.001$  in all cases). There was a significant effect of age on the caffeine induced improvement in  
275 power output (ANOVA  $p < 0.005$ ). The caffeine induced increase in power output at 10 weeks was the  
276 highest and was significantly greater than at 3, 30 and 50 weeks (Fig 2; Tukey  $p < 0.004$  in all cases).  
277 Power output was also significantly higher at 30 weeks compared to 3 weeks and 50 weeks (Fig 2;  
278 Tukey  $p < 0.02$  in both cases). There was no significant difference in power output between 3 weeks  
279 and 50 weeks (Fig 2; Tukey  $p = 0.864$ ).

280 The ergogenic benefit of 70 $\mu$ M caffeine treatment was significantly greater in EDL muscle compared  
281 to diaphragm at 30 and 50 weeks (Fig 3; two-sample t-test  $p < 0.005$  in both cases). The caffeine  
282 induced potentiation of diaphragm power output had a tendency to be greater than EDL at 10 weeks  
283 (two-sample t-test  $p = 0.054$ ). The caffeine induced increase in maximal muscle power output at 3  
284 weeks was not significantly different between muscles (independent samples t-test  $p = 0.54$ ).

285 *The Effect of 70 $\mu$ M Caffeine Treatment in Relation to Muscle Mass*

286 EDL muscle mass was significantly affected by age (ANOVA  $p < 0.001$ ). Muscle mass at 3 weeks  
287 ( $0.00672 \pm 0.00171\text{g}$ ), was significantly lower than that at 10 ( $0.01244 \pm 0.00038\text{g}$ ), 30 ( $0.01422 \pm$   
288  $0.00023\text{g}$ ), and 50 ( $0.00245 \pm 0.00078\text{g}$ ) weeks (Tukey  $p < 0.001$  in all cases). Muscle mass at 30 and  
289 50 weeks was significantly greater than that at 10 weeks (Tukey  $p < 0.001$ ). Similar comparisons for  
290 diaphragm cannot be made due to usage of only part of the diaphragm. There was no significant  
291 relationship between whole EDL muscle mass, or the mass of the diaphragm strip, used in the  
292 assessment of muscle mechanics and the performance enhancing benefit of caffeine for any of the  
293 experimental groups (Table 1;  $p > 0.07$  in all cases). Therefore, as all of our power output values are  
294 expressed relative to control muscle power output the variation in muscle mass between preparations  
295 should not need to be further accounted for in our analysis.

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308 **Discussion**

309 *The Direct Effect of 70 $\mu$ M Caffeine on Aged Skeletal Muscle*

310 The present findings are the first to demonstrate that 70 $\mu$ M caffeine can directly potentiate skeletal  
311 muscle power output across a broad range of ages and subsequently adds further evidence to the small  
312 body of human research that suggests that caffeine could be used as an effective performance  
313 enhancing nutritional supplement in older adult populations [20, 39]. The present work is the first to  
314 assess how the sensitivity of the caffeine effect on exercise changes with age. The results demonstrate  
315 that the magnitude of the performance enhancing effect is influenced by age and muscle fibre type  
316 composition. The caffeine induced potentiation of both EDL and diaphragm muscle power output was  
317 significantly reduced in the oldest and youngest age groups compared to the younger adult groups.

318 The caffeine induced increase in EDL muscle power output at 10 weeks of age (up to 4%) is  
319 consistent with the 3% reported by James et al, [28] and Tallis et al, [55] using similar experimental  
320 methods. However, caffeine treatment caused the greatest increase in muscle power output at 30  
321 weeks of age. This is particularly interesting given that the onset of the early age related decline in  
322 contractile performance has been demonstrate to occur at 30 weeks of age in this strain of mice (see  
323 [57] for a full report of the effect of increasing age on mechanical performance of skeletal muscle  
324 used in this study). The present work is the first to assess the direct effect of 70 $\mu$ M caffeine on  
325 diaphragm muscle and the 6% increase in muscle power is comparable to that previously reported for  
326 mouse soleus [55]. In mice both soleus and diaphragm have previously been shown to be composed of  
327 more aerobic fibres than EDL [1, 7]. Unlike in EDL, an increase in age beyond that associated with  
328 peak mechanical performance of diaphragm [57] caused a significant reduction in the magnitude of  
329 the caffeine effect.

330 This age related reduction in skeletal muscle caffeine sensitivity occurs independently of changes in  
331 muscle mass (Table 1). One may speculate that the same absolute dose of caffeine may provide less of  
332 a benefit to larger muscles (as in the older age groups) due to a smaller concentration per area of  
333 tissue, but this did not prove to be the case. Therefore, it seems likely that skeletal muscle ageing may

334 be accountable for this observed loss in the ergogenic effect in the oldest mice used in the present  
335 study.

336 The rate of muscle activation and force production is primarily determined by effectiveness of  
337 excitation contraction coupling and the intramuscular  $\text{Ca}^{2+}$  transient time [9]. The demonstrated  
338 caffeine induced increase in maximal power output is likely to occur due to the effects of caffeine as a  
339 modifier of excitation-contraction coupling [23, 33]. Mechanistically, it is believed that caffeine acts  
340 as a direct agonist to adenosine receptors on the skeletal muscle membrane and has been demonstrated  
341 to bind to ryanodine receptors (RyR) of the sarcoplasmic reticulum (SR) [10, 14, 15, 21, 46]. It is  
342 likely that these actions result in an improved opening of RyR channels promoting a greater  $\text{Ca}^{2+}$   
343 release into the intracellular space, an increased  $\text{Ca}^{2+}$  myofibrillar sensitivity, a decreased SR  $\text{Ca}^{2+}$   
344 pump sensitivity and increased SR  $\text{Ca}^{2+}$  permeability. Consequently a reduction in the rate of  $\text{Ca}^{2+}$   
345 efflux from the intracellular space back to the SR is likely to occur resulting in an elevated basal and  
346 subsequently activated intracellular  $\text{Ca}^{2+}$  concentration [2, 3]. Mechanical data from our previous  
347 work examining the effect of  $70\mu\text{M}$  caffeine on the maximal power output of isolated skeletal muscle  
348 supports this, demonstrating an increase in work during the shortening phase of the work loop [55],  
349 and an increase in relaxation time and subsequent work required to re-lengthen the muscle at the end  
350 of the shortening phase during fatigue testing [56].

351 The age related reduction in the contractile performance of the muscles in the present study is  
352 believed to result from a reduction in the function of excitation-contraction coupling, as no substantial  
353 changes in either EDL or diaphragm fibre type composition or metabolic capacity were found  
354 between these same age groups in a previous study [57]. Renganathan et al, [45] demonstrated a  
355 significant age related reduction in SR  $\text{Ca}^{2+}$  release of rat EDL and soleus muscle attributed to DHPR-  
356 RyR uncoupling. This was further supported by Delbono et al, [17] who confirmed a similar effect in  
357 fast fibres of human quadriceps muscle. A reduction in the voltage gated SR  $\text{Ca}^{2+}$  release mechanism  
358 would result in a decreased  $\text{Ca}^{2+}$  availability for the contractile proteins and consequently a reduction  
359 in contractile force. Therefore, in the context of the present study, caffeine may still bind to RyR in  
360 the aged muscle but the age related uncoupling of DHPR-RyR may result in a decrease in the force

361 and power enhancing effects of caffeine. In addition Larsson & Salviati [35] reported an age related  
362 reduction in SR  $\text{Ca}^{2+}$  concentration of fast twitch muscle of chemically skinned rats. As such it is  
363 likely that the reduction in the ergogenic properties of caffeine in aged muscle arise from a reduced  
364 ability to increase SR  $\text{Ca}^{2+}$  release.

#### 365 *The Direct Effect of 70 $\mu\text{M}$ Caffeine on Developing Skeletal Muscle*

366 Despite the high consumption of caffeine in children, and caffeine containing products marketed  
367 specifically at this age group, the effect of caffeine on exercise performance in this population is not  
368 well understood [5]. Turley, Bland and Evans [58] reported limited effects of low, mild and high dose  
369 caffeine (1, 3 and 5  $\text{mg}\cdot\text{kg}^{-1}$ ) on physiological responses to exercise in 7-9 year old children. Caffeine  
370 treatment had no effect on substrate metabolism, however, mild and moderate dose caffeine treatment  
371 resulted in a slight increase in blood pressure and a slight decrease in heart rate.

372 The present study shows that the treatment of muscle from 3 week old mice with 70 $\mu\text{M}$  caffeine has  
373 only a very small, but significant, effect on the potentiation of maximal work loop power output. The  
374 ergogenic benefit in this age group was not significantly different between diaphragm and EDL.  
375 Schiaffino and Margreth [48] and Luff and Atwood [36] established that at birth skeletal muscle SR is  
376 sparse and occurs as a loose network of tubes. Furthermore Luff and Atwood [36] demonstrated a  
377 muscle fibre type specific increase in mouse SR from 1.1% of fibre volume at birth to 5.5% in adult  
378 EDL (predominantly fast-twitch), and from 1.7% at birth to 2.9% in adult in soleus (predominantly  
379 slow-twitch). SR development occurred most rapidly in the initial 20 days and had reached adult  
380 values by 30 days in soleus and was still increasing after 60 days in EDL. Therefore it should be  
381 considered that in the 3-week-old diaphragm and EDL muscle used in present study, SR is not fully  
382 developed, thereby possibly limiting the action of caffeine on RYR and consequently reducing the  
383 level of caffeine induced  $\text{Ca}^{2+}$  release and subsequent force potentiating effects.

384 In line with previous literature the present work infers that caffeine has limited effect on physiological  
385 responses to exercise and skeletal muscle performance in a pre-mature age group. However the  
386 stimulant effects at the central nervous system should be fully evaluated *in vivo* before conclusions are



387 made about the value of caffeine as an ergogenic aid in children. Hughes and Hale [26] reported only  
388 slight improvements in vigilance performance and decreased reaction time in this population;  
389 however the effect of caffeine on the cognitive function of children requires further investigation.

#### 390 *The Muscle Specific Action of 70 $\mu$ M Caffeine Treatment*

391 At 10 weeks of age the caffeine induced potentiation of maximal skeletal muscle power output was  
392 significantly greater in diaphragm muscle compared to EDL. However, beyond this the effect of  
393 caffeine was greater in aged EDL. It has been firmly established in adult rodent models that the force  
394 potentiating effects of caffeine are more greatly pronounced in muscle with a predominantly slower  
395 fibre type [22, 46, 55]. Although of mixed muscle phenotype, diaphragm will consist of a significantly  
396 greater proportion of oxidative muscle fibres than EDL which subsequently may explain why the  
397 caffeine induced benefit was greater in this muscle at 10 weeks of age (Figure 3). One may therefore  
398 argue that the widely reported age associated increase in type I fibre expression [18] would actually  
399 increase caffeine sensitivity. The likely absence of an age related change in fibre type expression in  
400 the muscles used in the present study, and the reported significantly greater loss in mechanical  
401 function in the diaphragm [57], likely explain why caffeine sensitivity was more dramatically reduced  
402 in this muscle.

403 Irrespective of age the present study is the first to examine the direct effect of a physiologically  
404 relevant dose of caffeine on the mechanical performance of the diaphragm. Kivity et al, [34]  
405 demonstrated that high dose caffeine significantly improved the pre and post exercise forced  
406 expiratory volume in one second (FEV1) in healthy human male subjects, which was largely  
407 attributed to caffeine induced bronchodilation. In alignment with the work of Brinbaum and Herbst  
408 [11], these findings suggest that caffeine may evoke a significant increase in respiratory function  
409 across a range of ages which will have profound effects on exercise capacity by working to meet the  
410 oxygen demand of the active tissue. Our findings, combined with those of the previous studies suggest  
411 that caffeine may be effective in reducing the functional strain on patients with age associated  
412 respiratory diseases such as COPD.

413 *Practical Implications and Limitations of the Present Findings*

414 The present findings have used a more controlled approach to demonstrate that caffeine could be used  
415 as an effective nutritional supplement to directly improve muscle function across a broad range of  
416 ages. Although the magnitude of the effect was reduced in the older age groups, this small but  
417 significant benefit could still be effective in evoking meaningful improvements in functional  
418 performance. When the *in vivo* applications are considered, such increases in muscle power output are  
419 likely to transfer to an improved resistance to fatigue as caffeine treated muscles will be able to  
420 produce the same relative power output with a smaller number of recruited fibres.

421 In line with our previous work the present results demonstrates an individual response to the caffeine  
422 treatment ranging between a 0-11% increase in power in the young adult group [55], however, the  
423 present study shows a lower effect, 0-5%, in the older adult group. These findings are particularly  
424 interesting given that individual responses have, in human literature, been attributed to caffeine  
425 habituation [49]. As this factor is not prevalent in a rodent model it appears that the individual  
426 responses to the performance enhancing effect of caffeine needs further investigation. The present  
427 results imply that there is no 'one size fits all' approach for maximising the caffeine response as some  
428 individuals may have a large increase in performance whilst others demonstrate little or no effect,  
429 regardless of previous caffeine exposure.

430 In addition, caffeine has been shown to promote performance enhancing effects through its action as a  
431 central nervous system stimulant across a range of ages [23, 44]. This coupled with the increase in  
432 skeletal muscle performance reported in the present study could evoke more substantial increases in  
433 functional performance than that demonstrated by our present approach of examining the direct  
434 effects on skeletal muscle alone.

435 Conceptually there is difficulty in relating the 70 $\mu$ M caffeine concentration used in the present work  
436 to quantities of oral caffeine consumption due to individual differences in the rate of caffeine  
437 metabolism [51], and the need to administer caffeine relative to body mass rather than as an absolute  
438 dose. Graham [23] stated that normal plasma caffeine concentrations range between 20-50 $\mu$ M and

439 Van Soeren and Graham [59] demonstrated that 6 mg/kg caffeine consumption resulted in blood  
440 plasma level of ~50 $\mu$ M in athletes aged 37 years. Such moderate concentrations are used regularly in  
441 human literature, thus it is conceivable that a blood plasma concentration of 70 $\mu$ M can be achieved  
442 using high, but safe oral doses of caffeine. Our previous work using 10 week old mice has shown that  
443 caffeine induced improvements in contractility demonstrated at 70 $\mu$ M are still prevalent at 50 $\mu$ M  
444 [53]. There is no reason to assume that similar doses will not be equally effective in this ageing  
445 model.

446 Despite findings demonstrating the benefits of caffeine consumption, the older adult population  
447 should exercise caution when consuming high quantities of caffeine. In some individuals, high doses  
448 of caffeine can lead to increased anxiety, gastrointestinal discomfort, and impairment of fine motor  
449 control [12, 50]. Evidence has demonstrated that daily consumption of high doses of caffeine will  
450 accelerate bone mineral density (BMD) loss in healthy postmenopausal women with calcium intakes  
451 below the recommended daily intake [25], however this has not been associated with an increased risk  
452 of bone fracture [24]. Although caffeine may increase blood pressure in non-habitual users, recent  
453 evidence demonstrated that heavy coffee consumption is not associated with an increased risk of  
454 coronary heart disease and that moderate consumption may actually negate this risk [13, 19].  
455 Evidence further suggests that caffeine may have protective effects on late life cognitive decline,  
456 dementia [47, 42] and can act as a therapeutic tool in Parkinson's disease [43].

457 The present work examines the caffeine response during early ageing, however future work should  
458 investigate skeletal muscle responses at the later extremities of older ageing. Although it may be  
459 considered that the performance enhancing benefit would decrease if the current trend is continued, an  
460 age related shift to a slower muscle fibre type composition could mitigate such a response.

#### 461 *Conclusion*

462 The methods employed in the present study have allowed a more controlled investigation of how the  
463 direct performance enhancing effect of caffeine is affected by increasing age. Our results demonstrate  
464 that the direct treatment of skeletal muscle with, physiologically relevant, 70 $\mu$ M caffeine significantly

465 potentiated the maximal power output of mouse EDL and diaphragm muscle independent of age. As  
466 such, caffeine may be used as an effective performance enhancing nutritional supplement in an older  
467 adult population. This is particularly significant given the equivocal findings reported in the small  
468 quantity of whole body human studies examining the effects of caffeine in the elderly. Interestingly  
469 however, our results are the first to demonstrate that the ability of caffeine to produce such effects is  
470 significantly reduced in this older age group, when compared to younger adults, which is likely to be  
471 related to the age related reduction in muscle function caused by a reduction in the efficiency of the  
472 excitation contraction coupling process.

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648 **Figure Legends**

649 Figure 1 – The effect of 70 $\mu$ M caffeine on the mean acute maximal power output of mouse EDL  
650 isolated from 3, 10, 30 and 50 week old mice [Data represented as mean  $\pm$  SE: n=10 in each case].

651 Figure 2 – The effect of 70 $\mu$ M caffeine on the mean acute maximal power output of mouse diaphragm  
652 isolated from 3, 10, 30 and 50 week old mice [Data represented as mean  $\pm$  SE: n=10 in each case].

653 Figure 3 - Comparison of the peak effect of caffeine on muscle power output, with increased age,  
654 between EDL and diaphragm [Each data point represented as mean  $\pm$  SE: n=10 in each case].

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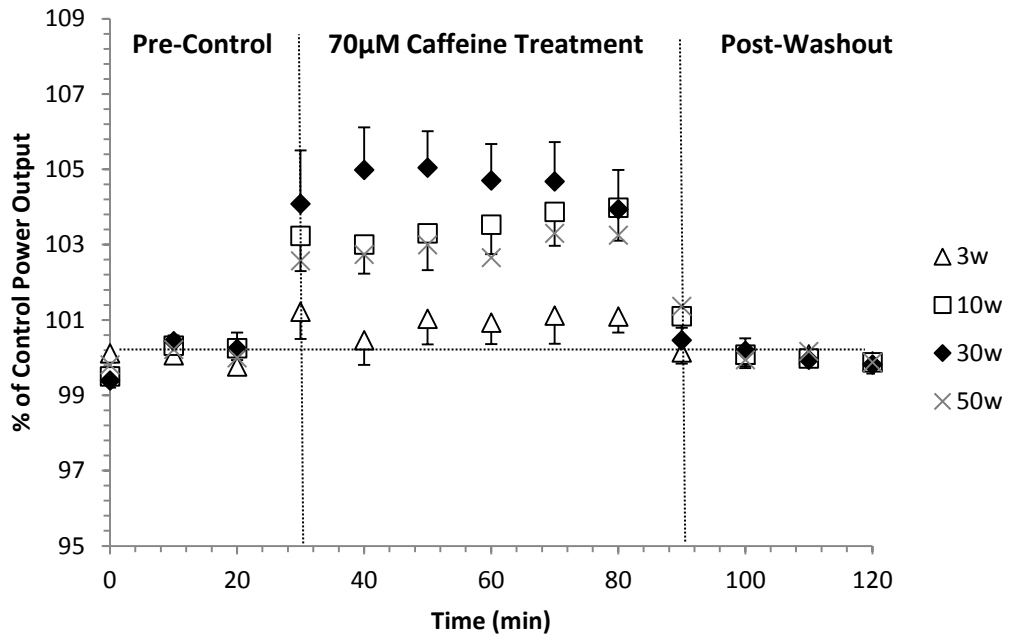
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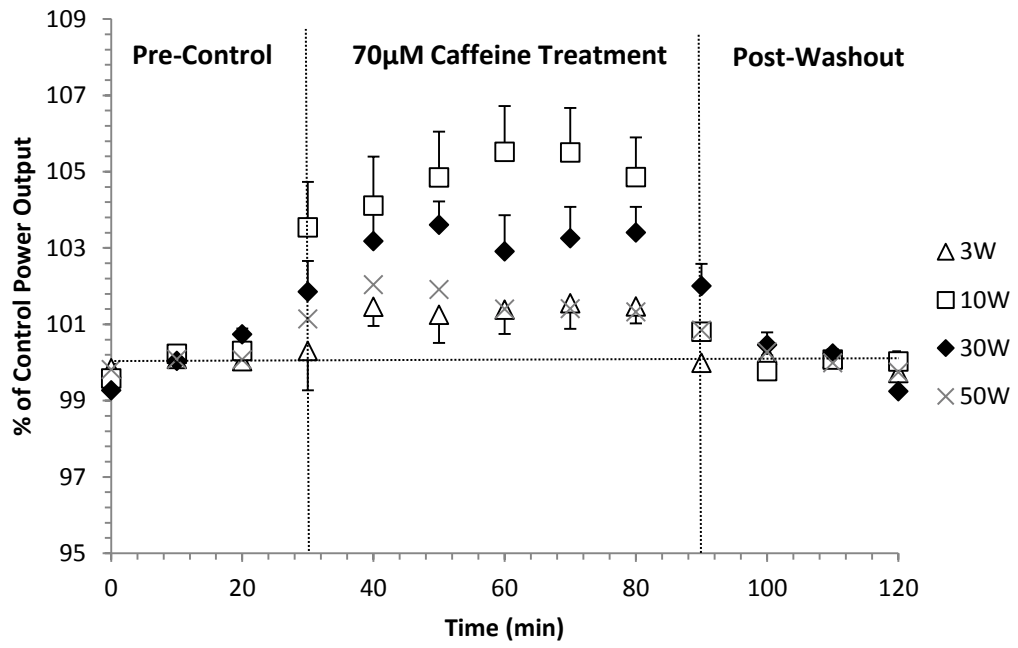
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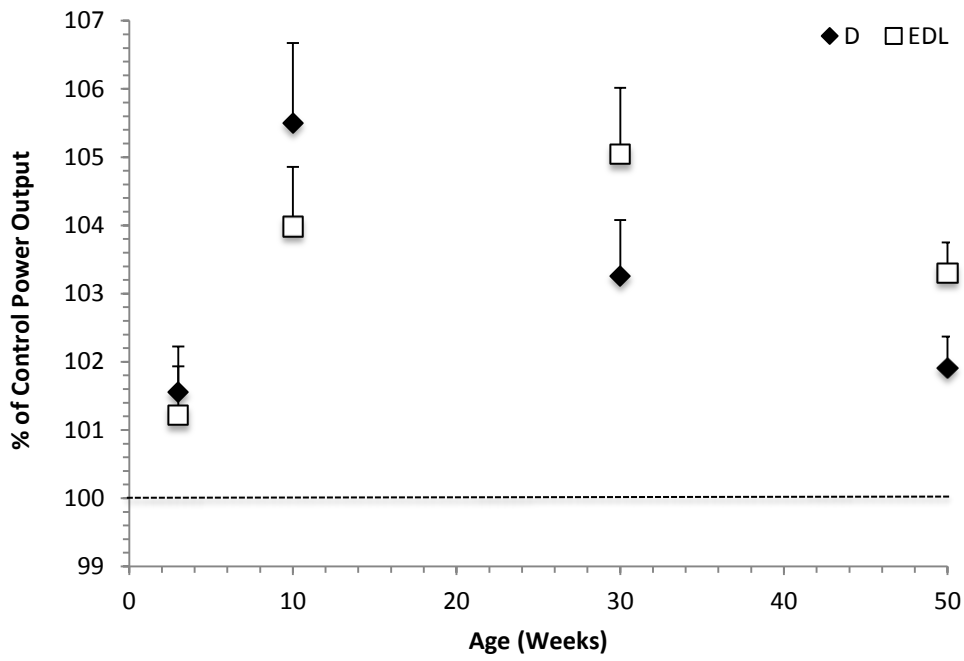
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**Table 1 – There was no relationship between caffeine treated peak power output and muscle mass**

<b>Animal</b>	<b>3 Wk Mmass (g)</b>	<b>3 Wk PPO (%)</b>	<b>10 Wk Mmass (g)</b>	<b>10 Wk PPO (%)</b>	<b>30 Wk Mmass (g)</b>	<b>30 Wk PPO (%)</b>	<b>50 Wk Mmass (g)</b>	<b>50 Wk PPO (%)</b>
<i>EDL</i>								
<b>1</b>	0.0077	103.15	0.0126	110.67	0.0137	103.96	0.018	103.96
<b>2</b>	0.0076	100.39	0.012	106.11	0.0136	109.21	0.0162	103.37
<b>3</b>	0.0099	101.79	0.0129	100.63	0.014	104.76	0.0165	101.75
<b>4</b>	0.0067	106.05	0.0133	107.89	0.0159	103.19	0.0152	103.78
<b>5</b>	0.0085	103.02	0.0132	102.76	0.0142	105.82	0.0214	104.64
<b>6</b>	0.0061	101.23	0.0111	105.12	0.0146	102.21	0.0155	104.06
<b>7</b>	0.0048	102.00	0.0127	102.96	0.0143	107.57	0.0133	104.85
<b>8</b>	0.0044	99.84	0.0098	102.59	0.0142	100.74	0.0137	101.44
<b>9</b>	0.0058	100.45	0.0141	105.70	0.0145	110.06	0.0167	103.28
<b>10</b>	0.0057	103.11	0.0127	102.93	0.0132	106.98	0.0134	105.48
<i>r value</i>	0.233		0.168		-0.323		0.052	
<i>P value</i>	0.516		0.642		0.362		0.887	
<i>Diaphragm</i>								
<b>1</b>	0.0115	98.10	0.0176	111.52	0.0225	103.63	0.0369	101.80
<b>2</b>	0.0098	104.88	0.0157	102.35	0.0147	108.63	0.0308	101.86
<b>3</b>	0.0081	101.65	0.0187	101.65	0.015	102.48	0.0321	102.26
<b>4</b>	0.0078	103.58	0.0132	106.67	0.024	103.78	0.0226	103.24
<b>5</b>	0.0087	102.12	0.0131	106.20	0.0233	103.84	0.0298	98.78
<b>6</b>	0.005	102.80	0.012	112.02	0.0251	104.40	0.025	103.51
<b>7</b>	0.0071	101.14	0.0155	104.12	0.0264	103.99	0.0276	101.56
<b>8</b>	0.0047	103.89	0.016	107.38	0.0281	102.60	0.0203	104.24
<b>9</b>	0.0078	101.77	0.0159	104.95	0.0119	103.81	0.0264	101.77
<b>10</b>	0.0066	101.90	0.0171	101.80	0.0195	100.66	0.022	103.51
<i>r value</i>	-0.465		-0.459		-0.231		0.593	
<i>P value</i>	0.176		0.183		0.521		0.071	