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1 **The Early Effects of Ageing on the Mechanical Performance of Isolated Locomotory (EDL)**  
2 **and Respiratory (diaphragm) Skeletal Muscle Using the Work Loop Technique**

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9 Effect of Ageing on Mechanical Properties of Skeletal Muscle

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21 **Abstract**

22 Previous isolated muscle studies examining the effects of ageing on contractility have used  
23 isometric protocols which have shown to have poor relevance to dynamic muscle  
24 performance that occurs *in vivo*. The present study uniquely uses the work loop technique to  
25 obtain a more realistic estimation of *in vivo* muscle function in order to examine changes in  
26 mammalian skeletal muscle mechanical properties with age. Measurements of maximal  
27 isometric stress, activation and relaxation time, maximal power output, sustained power  
28 output during repetitive activation and recovery are compared in locomotory EDL and core  
29 diaphragm muscle isolated from female mice 3, 10, 30 & 50 weeks old to examine the early  
30 onset of ageing. A progressive age related reduction in maximal isometric stress that was of  
31 greater magnitude than the decrease in maximal power output, occurred in both muscles.  
32 Maximal force and power developed earlier in diaphragm muscle compared to EDL, but  
33 demonstrated a greater age related decline. The present study indicates that ability to  
34 sustain skeletal muscle power output through repetitive contraction is age and muscle  
35 dependent, which may help to rationalise previously reported equivocal results examining  
36 the effect of age on muscular endurance. The age related decline in EDL muscle  
37 performance is prevalent without a significant reduction in muscle mass, and biochemical  
38 analysis of key marker enzymes suggest that although there is some evidence of a more  
39 oxidative fibre type, this is not the primary contributor to the early age related reduction in  
40 muscle contractility.

41 **Key Words:** Ageing, Fatigue, Power, Sarcopenia, Work Loop

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45 **Introduction**

46 The age related reduction in skeletal muscle function has been studied at length and is  
47 primarily associated with a loss of muscle mass, strength and a slowing of contractile  
48 function that greatly reduces mobility and subsequently the quality of life in elderly  
49 populations [72]. However, muscle atrophy and associated decline in skeletal muscle  
50 performance can occur as early as 25 years of age in humans, and is greatly accelerated at  
51 later stages of life [42]. It is impossible to fully offset the age related decline in muscle  
52 function and changes in body composition, even with a physically active lifestyle [37]. Little  
53 is known about the rate of decline in muscle performance between peak performance and  
54 'old age'. Hence, the present study aims to assess the mechanical properties of mammalian  
55 skeletal muscle during early development and at various stages after physiological maturity  
56 to determine the time course of early age-related declines in muscle performance.

57 Evidence demonstrating an age related reduction in muscle strength (maximal force in a  
58 single attempt) and power (the rate at which work is done; the product of force produced  
59 and the speed of muscle shortening) is commonplace in whole body human research [20,  
60 21, 48, 50, 51]. It is further considered that the decline in muscle power occurs significantly  
61 faster than the loss of strength, which is partly attributed to a reduction in the muscle force-  
62 velocity relationship and maximal unloaded shortening velocity [38, 48, 57]. With *in vivo*  
63 mammalian research it is difficult to ascertain the true extent of the direct effect of ageing  
64 on skeletal muscle mechanical performance, whereas *in vitro* isolated muscle studies on  
65 rodents, have further demonstrated a muscle specific, age related reduction in maximal  
66 force [14, 25, 66, 73]. Although there is some *in vitro* evidence of a greater reduction in  
67 muscle power [45], this area of research is relatively sparse, and the estimation of muscle  
68 power from 'iso' testing methods has poor *in vivo* relevance [33, 34]. Furthermore, Brooks  
69 and Faulkner [14] demonstrated a reduction in the muscle specific force of mouse EDL

70 without changes in the force-velocity relationship, and hence the assessment of changes in  
71 muscle power output with age requires further investigation.

72 Studies investigating the effect of increasing age on muscular endurance (the ability of the  
73 muscle to resist sustain performance over time) have demonstrated equivocal findings in  
74 both *in vivo* human [10, 11, 30, 40, 43] and isolated mammalian muscle research [16, 24, 55,  
75 73]. Discrepancies in results are at least partly due to variations in experimental methods.  
76 Namely differences are apparent in the protocols used to determine the ability fo muscle to  
77 sustain performance, the duration for which muscle endurance is measured, and the muscle  
78 groups tested [20]. Zhang and Kelsen [73] reported a reduced fatigue resistance of isolated  
79 diaphragm strips from 18 month old hamsters stimulated via repeated isometric tetanic  
80 contraction. In contrast, González and Delbono [24] concluded that the reduction in  
81 mechanical performance was not related with changes in fatigability of EDL and soleus  
82 muscle taken from 20-24 month mice. Further ambiguity is added by examining the findings  
83 of Pagala et al [55], who reported that despite a decline in whole animal endurance  
84 performance in aged mice (34-36 months), the fatigue resistance of tetanic stress (force per  
85 cross-sectional area) was significantly increased. The research outlined here has assessed  
86 the ability of muscle to sustain performance via repeated tetanic contraction which is a poor  
87 indicator of dynamic skeletal muscle action *in vivo* [35]. Furthermore, there is a distinct lack  
88 of evidence exploring the effect of age on the maintenance of muscle power output during  
89 repetitive activity.

90 The present study uniquely uses the work loop technique as a more realistic estimation of *in*  
91 *vivo* muscle function in order to examine changes in mammalian skeletal muscle mechanical  
92 properties with age [34,35]. Isometric contractions are relatively rare *in vivo*, and this may  
93 result in discrepancies when relating findings of previous ageing work [14, 16, 23, 24, 25, 34,  
94 35] to whole animal performance. There is a dearth of *in vitro* studies examining the effect

95 of ageing on muscle power, and estimations of power output from isometric and isoveloc  
96 data, as Lynch et al [45], are poor estimates of power obtained by the work loop [31, 34].  
97 Furthermore, skeletal muscle cannot shorten indefinitely and must, at some stage, go  
98 through a period of lengthening before subsequent contraction. As for *in vivo* power  
99 producing muscles, the work loop technique considers muscle force production over  
100 dynamic contractions accounting for the interaction of force production during shortening,  
101 resistance to muscle re-lengthening and changes in activation and relaxation time using  
102 waveforms and stimulation parameters that more closely replicate those used *in vivo* [31,  
103 32, 34, 35].

104 Much of the ageing research measuring skeletal muscle activity in rodents compares a  
105 physiologically mature population against an aged population and relatively little is known  
106 about the rate of decline between these extremities. The present study implements the  
107 work loop method to determine the time course of age related changes in mechanical  
108 properties of mouse EDL (typically IIx 9.3%, IIB 86.8%, I 3.9% at 90 days [1]) and diaphragm  
109 (typically IIa 43.6% IIx 34.6%, IIB 6.2%, I 15.6% at 90 days [1]) muscles. It is hypothesised that  
110 significant detrimental changes in: 1) maximal isometric force and dynamic power output; 2)  
111 muscle activation and relaxation time; 3) ability to sustain muscle power output through  
112 repetitive activation; 4) post sustained activity recovery will occur well in advance of 'old  
113 age' and that the decline in performance will be muscle and age specific. It is further  
114 considered that diaphragm muscle will develop more quickly in early life and will maintain  
115 greater mechanical function in older age groups due to its underlying functional significance.  
116 The reduction in fast muscle fibre types is commonplace in ageing skeletal muscle [5, 7, 17],  
117 and thus it is considered that EDL will age more quickly. In conjunction with this, biochemical  
118 analysis of key marker enzymes will support a reduction in muscle anaerobic glycolysis and  
119 oxidative capacity with ageing, with the former being more greatly pronounced in EDL.

120 **Materials and Methods**

121 *Animals*

122 The ethics committee of Coventry University approved the use of animals in this study.  
123 Conventionally kept female mice (strain CD1, Charles River, UK), not in specific pathogen  
124 free (SPF) conditions, were bred and kept at Coventry University. All animals were kept in  
125 groups of 8-10 in 12:12-h light-dark cycle and supplied with food (CRM(P); SDS/Dietex  
126 international Ltd) and water ad libitum.

127 From birth, mice were housed in groups of 8 without access to running wheels and were  
128 sampled at 3 weeks, 10 weeks, 30 weeks, and 50 weeks of age (n = 20 for each age group).  
129 Pups were weaned 21 days postpartum, at this age animals are significantly smaller than  
130 those at 10 weeks where they are believed to be adult. Hence muscle dissected from 3 week  
131 old mice were used to represent growth. Lang and White [39] demonstrated a survival rate  
132 above 85% for CD1 mice at 50 weeks of age, however beyond this point mortality rate  
133 increased more rapidly and at 18 months was approximately 50%. Previous research  
134 examining the ageing effect on skeletal muscle mechanical performance has commonly used  
135 18-24 month old mice from different strains (C57BL/6, DBA, FVB) to represent 'old age' [14,  
136 23, 24, 25]. Similarly, 18-24 month CD-1 mice have been used as animal models for ageing  
137 research (Strochacker et al, 2012; Warrington et al, 2000; 2003). 12 month old mice were  
138 used to represent a 'middle aged' group by Gonzalez et al. [25] who investigated the  
139 reduction in specific force of EDL and soleus muscle fibres. In light of this and the work by  
140 Lang and White [39], mice at 50 weeks of age were used in the present study to represent a  
141 mature group. Assessment of mechanical performance was also conducted at 30 weeks of  
142 age to represent a development group, in an attempt to not only assess the early onset of  
143 ageing, but to examine if a decline in performance was linear. Mice from each age range  
144 were tested within 7 days of reaching their target age.

145 *Dissection*

146 Mice were killed by cervical dislocation in accordance with British Home Office Animals  
147 (Scientific Procedures) Act 1986, Schedule 1 and then weighed to determine body mass. EDL  
148 muscle was dissected from the right hind limb, and pinned out to approximately its resting  
149 length at room temperature (19-21°C). Throughout the dissection procedure the muscle was  
150 maintained in refrigerated and frequently changed oxygenated (95% O<sub>2</sub>; 5% CO<sub>2</sub>) Krebs-  
151 Henseleit solution of composition (mM) NaCl 118; KCl 4.75; MgSO<sub>4</sub> 1.18; NaHCO<sub>3</sub> 24.8;  
152 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.  
153 Aluminium foil T-clips were wrapped around each tendon to minimise tendon slippage  
154 during muscle force production. Whole diaphragm muscle was dissected out, but only a  
155 ventral section of the costal diaphragm was used in the muscle mechanics protocol;  
156 aluminium foil T-clips were wrapped around the central tendon at one end, and at the  
157 opposing end two ribs anchoring the muscle were left intact.

158 EDL muscle and diaphragm muscle were dissected from another animal from the same age  
159 in the same manner but were immediately frozen in liquid nitrogen for later biochemical  
160 analysis.

161 *Measurement of Mechanical Properties*

162 Each muscle preparation was placed in a flow-through chamber and the foil clips or bone  
163 were used to attach the muscle, via crocodile clips, to a force transducer at one end (UF1,  
164 Pioden Controls Ltd, UK) and at the opposing end to a motor (V201, Ling Dynamic Systems,  
165 UK). Position of the motor arm was detected via a Linear Variable Displacement Transformer  
166 (DFG5.0, Solartron Metrology, UK). Unlike in previous research examining the direct skeletal  
167 muscle ageing effect where a much lower temperature was used [14, 23, 24, 25], the muscle  
168 was maintained in circulated oxygenated Krebs-Henseleit solution at a constant 37±0.5°C to



169 represent *in vivo* physiological temperature, as used in our previous work [31, 64, 65]. The  
170 muscle was activated via electrical stimulation through parallel platinum electrodes that lay  
171 inside the muscle chamber. These electrodes were not in contact with the nerve branch or  
172 the fibre itself but stimulated the muscle via the surrounding fluid. Muscle stimulation and  
173 length changes were controlled using custom written software (Testpoint, CEC,  
174 Massachusetts, USA) via a D/A board (KPCI3108, Keithley Instruments, Ohio, USA) on a  
175 standard desktop PC.

176 Each muscle preparation was electrically stimulated whilst held at a constant length in order  
177 to produce a series of twitch responses. Muscle length and stimulus amplitude (14-18V for  
178 EDL; 10-16V for Diaphragm; current fixed at 160 mA) were optimised in order to achieve  
179 maximal isometric twitch force. The muscle length that corresponded to maximal twitch  
180 force was measured; using an eye piece graticule fitted to a microscope, and was defined as  
181  $L_0$ . Mean muscle fibre length was calculated as 75% of  $L_0$  for EDL muscle [31], as in a number  
182 of previous publications examining the mechanical performance of mouse EDL [28, 31, 32].  
183 As no such estimate of fibre length exists for diaphragm the physical measurement taken  
184 was used as  $L_0$ . Maximal isometric tetanic force was measured by subjecting each muscle  
185 preparation to a 250ms burst of electrical stimulation. The frequency of stimulation was  
186 further optimised in each muscle order to yield maximal tetanic force; this was usually  
187 200Hz for EDL, 140Hz for diaphragm and did not change with age. Time to half peak tetanus  
188 (THPT) and time from last stimulus to half tetanus relaxation (LSHR) were measured as  
189 indicators of muscle activation and relaxation time. A 5-minute rest period was imposed  
190 between each tetanus in order to allow sufficient time for the muscle to recover.

191 All EDL and diaphragm muscle followed this process of isometric measures before  
192 proceeding on to the subsequent work loop protocol. Here the muscle was held at the  
193 previously determined  $L_0$  and the stimulation amplitude and frequency parameters that

194 were optimised to yield maximal tetanic force were employed. Each muscle was subjected  
195 to four sinusoidal length change cycles per set at a total symmetrical strain of 0.10 around  
196 the previously determined  $L_0$ . A cycle frequency of 10Hz and 7Hz was used for EDL and  
197 diaphragm muscle respectively. 10Hz represents the cycle frequency that has been  
198 previously shown to elicit maximal power output in isolated mouse EDL [31]. 7Hz was the  
199 cycle frequency found to elicit maximal power concurrent with the findings of Altringham  
200 and Young [6]. The strain of 0.10 was based on previous estimations of the strain required  
201 for production of maximal power in both EDL and diaphragm [6, 31]. For EDL a strain of 0.10  
202 is attainable during *in vivo* locomotion [31]. The magnitude and frequency of length changes  
203 and electrical stimulations were controlled via the Testpoint software. Data were sampled at  
204 a rate of 10 kHz and then a work loop was formed, by plotting force against length, the area  
205 of which represents the net work done by the muscle during a single length change cycle  
206 [35]. The preparations were electrically stimulated by altering burst duration (amount of  
207 stimulation through muscle shortening) until maximal net power output was achieved.

208 As in the study by James et al. [31], a 49ms burst duration was used to elicit maximal power  
209 output at 10Hz cycle frequency. The burst duration to elicit maximal muscle power in  
210 diaphragm muscle was usually 55ms. On occasions the burst duration had to be altered to  
211 adjust the number of stimuli given in order to maximise muscle power output of individual  
212 muscle preparation. This alteration in burst duration was determined by examining the  
213 maximal work loop power output and by interpretation of the work loop shapes. i.e. if the  
214 muscle is too active during re-lengthening it will significantly distort the shape of the loop  
215 and reduce muscle power output by increasing the resistance of the muscle to stretch. A  
216 stimulation phase shift of -2 ms and -5 ms were used for EDL and diaphragm respectively as  
217 they elicited maximal power output. Such stimulation phase shifts dictate that stimulation of  
218 the muscle starts 2 ms (in EDL) or 5 ms (in diaphragm) prior to the muscle reaching maximal  
219 length.

220 Each muscle was subjected to four sinusoidal length change cycles at 10-minute intervals  
221 until maximal muscle power output was achieved. The third work loop of each set of four  
222 typically produced the highest power and was therefore taken as the indicative measure of  
223 muscle power output in all work loop experiments. A 10-minute rest interval was imposed  
224 between each set of four work loops in order to allow the muscle sufficient recovery time.

225 *Sustained Work Loop Power:* In order to examine the age related effect on ability to sustain  
226 power output over repetitive activity, each muscle was subjected to 50 consecutive work  
227 loop cycles using the stimulation and length change parameters that elicit maximal power  
228 output. Data were recorded for every second loop until force had significantly reduced and a  
229 plateau occurred, or until net negative work was produced.

230 *Recovery from Repetitive Work Loop Activation:* The ability of the muscle to recover from  
231 repetitive work loop stimulation was monitored for 30 minutes. Three measurements of  
232 maximal work loop power output were made every 10-minutes and were compared directly  
233 to maximal muscle power output prior to the repetitive muscle activation protocol.

234 The experimental protocol was 230 minutes in duration, and control runs were performed  
235 regularly to monitor muscle performance over time. After 180 minutes, at the start of the  
236 repetitive work loop contraction protocol; muscle power output was still at  $86.2 \pm 2\%$  and  
237  $84.6 \pm 1.7\%$  of its maximal for EDL and diaphragm respectively. This indicated the quality of  
238 the muscle preparations was maintained through the duration of the experimental protocol.

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

240 At the end of the experiment the muscle was rapidly disconnected from the apparatus and  
241 the tendons and bones removed, leaving the muscle intact. Following this, the muscle was  
242 blotted on tissue paper to remove excess fluid. The muscle was then placed on an electronic  
243 balance (Mettler Toledo B204-S, Zurich, Switzerland) to determine wet mass. Immediately

244 after this the muscle was frozen in liquid nitrogen, forming a second frozen tissue sample of  
245 that muscle from the same animal. Mean muscle cross-sectional area was calculated from  $L_0$ ,  
246 muscle mass and an assumed muscle density of  $1060 \text{ kg m}^{-3}$  [47]. Isometric stress was  
247 calculated as force divided by mean muscle cross-sectional area. Muscle power output was  
248 normalised to muscle mass to express power as  $\text{Watts.kg}^{-1}$ .

#### 249 *Biochemical analysis*

250 Maximal activities of lactate dehydrogenase (LDH), citrate synthase (CS) were measured,  
251 which represent marker enzymes for maximal glycolytic ATP production potential, and  
252 mitochondrial capacities, respectively. Furthermore the maximal activity of the sarco-  
253 endoplasmic reticulum  $\text{Ca}^{2+}$ -ATPase (SERCA) was determined, which is an important  
254 regulator of calcium resequestration into the sarcoplasmic reticulum and therefore muscle  
255 contraction-relaxation dynamics. Enzyme activities were determined according to published  
256 protocols [33, 59].

257 mRNA transcript content of RYR and fast and slow isoforms of SERCA and troponin were  
258 measured in order to determine if an age related change in skeletal muscle mechanical  
259 performance was related to changes in the quantity of these important mediating proteins  
260 in  $\text{Ca}^{2+}$  release, force production, and  $\text{Ca}^{2+}$  reuptake. As such, the biochemical analyses may  
261 offer insight into the interaction between quantity and dysfunction of these important  
262 proteins.

263 RNA was extracted from EDL and diaphragm muscle samples using TRI Reagent (Molecular  
264 Research Center, Cincinnati, OH, USA), following the manufacturer's instructions. RNA  
265 concentration and quality were verified using a spectrophotometer (NanoDrop  
266 Technologies, ThermoScientific, USA). An 800 ng sample of total RNA was treated with  
267 DNase I (Sigma) and reverse-transcribed using RNase H-MMLV reverse transcriptase

268 (Bioscript, Bioline, Australia) and random hexamer primers (Bioline, Australia). Quantitative  
269 RT-PCR was performed on an Applied Biosystems 7500 qRT-PCR machine (Applied  
270 Biosystems, Scoresby, VIC, Australia) according to published protocols [69].

271 Pre-validated TaqMan® Gene Expression Assays (Applied Biosystems, Australia) were used  
272 according to the manufacturer's instructions to quantify troponin 1 (tnni1; assay ID:  
273 Mm00502426\_m1), troponin 2 (tnni2; Mm00437157\_g1), Ca<sup>2+</sup>-transporting-ATPase 1  
274 (atp2a1; Mm01275320\_m1), Ca<sup>2+</sup>-transporting-ATPase 2 (atp2a2; Mm01201431\_m1),  
275 ryanodine receptor 1 (ryr1; Mm01175211\_m1) and elongation factor 1 $\alpha$ 2 (elf1a2;  
276 Mm00514649\_m1) expression, with Elf1a2 as the housekeeping gene. We used Taqman  
277 Gene Expression Mastermix (Applied Biosystems, Australia) with the standard PCR protocol  
278 as recommended by the manufacturer. The cycle consisted of 95°C for 7 min, 40 cycles of  
279 95°C for 20 s, 60°C for 1 min.

#### 280 *Statistical Analysis of Data*

281 Data are presented as means  $\pm$  s.e.m. Datasets were analysed by permutation analysis of  
282 variance (PERMANOVA; Primer 6 PRIMER-E Ltd, Plymouth, UK) using mouse muscle and age  
283 as the main factors and 10 000 permutations per run. We chose permutational analysis  
284 because it uses the data per se for statistical inferences rather than making assumptions  
285 about underlying distributions of the data; this is preferable for relatively small datasets  
286 [22].

287 In order to examine the effects of age on ability to sustain power, a PERMANOVA was  
288 conducted to examine the differences in work loop power at each stage of the protocol for  
289 each muscle tested. Comparisons were made until a reduction in muscle power output  
290 exceeded 50% compared to pre repetitive activation values. In order to assess whether  
291 recovery from repetitive activation was affected by age, we compared power output

292 between the different age groups at the final measurement of the recovery period (i.e. after  
293 30 min recovery) with a one-way PERMANOVA.

294 Results were interpreted as significant when  $p < 0.05$ . Values are displayed as mean  $\pm$   
295 standard error. In case of significant PERMANOVA results, we used *post hoc* pairwise tests to  
296 compare specific age groups.

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## 312 Results

### 313 *Body & Muscle Mass*

314 Increasing age resulted in a significant increase in mean mouse body mass (Fig 1A;  
315 PERMANOVA d.f. = 3, 76; F = 69.4; p <0.01). Whole animal body mass increased significantly  
316 (Fig 1A; pair-wise t > 9; p <0.01) at each age group and was greatest at 50 weeks (Fig 1A; t  
317 >5.5; pair-wise p <0.01 in all cases). At 50 weeks of age individual body masses had either  
318 increased above 70g (Fig 1A; X) or stayed below 50g (Fig 1A; Y), with this latter group similar  
319 to the mean body mass at 30 weeks of age. The distribution of the body masses between  
320 examined animals only permitted the analysis on the effects of skeletal muscle mechanical  
321 properties of diaphragm from 50-week-old obese (Fig 1A; X) and lean (Fig 1A; Y) mice (n=5  
322 each case). Despite no significant statistical difference in mean isometric stress ( $1.49 \pm 0.2$   
323 and  $1.44 \pm 0.13$  kN m<sup>-2</sup> for group X and Y respectively) and work loop power output (pair-wise  
324 t < 2; p > 0.17 in both cases), dynamic power output (expressed as Watts per kg muscle mass)  
325 for the lean group was 25% greater than the obese group.

326 For EDL, muscle mass was significantly affected by age (Table 1; Fig 1B; PERMANOVA d.f. = 3,  
327 36; F = 56.3 p <0.001). Mean muscle mass at 3 weeks of age was significantly lower than all  
328 other age groups (Table 1; Fig1B; pair-wise t >7; p <0.001 in all cases). Maximum muscle  
329 mass occurred in animals aged 50 weeks of age, which was 29% and 13% greater than at 10  
330 and 30 weeks respectively (Table 1; Fig1B; pair-wise t >4.2; p <0.001). Similar measures were  
331 not compared for diaphragm as only a section of the muscle was used to measure  
332 mechanical performance and hence the dissection affected the size of the muscle  
333 preparation.

### 334 *Maximal Isometric Twitch Stress*

335 Mean twitch stress was significantly affected by age (Fig 2A; PERMANOVA d.f. =79; F = 7.9; p  
336 =0.002). EDL twitch stress was greatest in 10 week old mice and was significantly lower at 3  
337 (by 39%), 30 (by 20%) and 50 (by 27%) weeks of age (Fig 2A; pair-wise t >2; p <0.01 in all  
338 cases). EDL twitch stress at 30 weeks was significantly higher than that at 3 weeks (Fig 2A;  
339 pair-wise t =2.4; p =0.026 in all cases). Absolute force values are provided in Table 1.

340 The mean twitch stress of diaphragm muscle was greatest in 10 week old mice and was  
341 significantly lower at 30 (by 34%) and 50 weeks of age (by 27%) (Fig 2A; pair-wise t >2.6;  
342 p<0.02 in both cases). Mean diaphragm twitch stress at 3 weeks had a tendency to be  
343 greater than that at 30 weeks (Fig 2A; pair-wise t =2; p =0.05).

#### 344 *Maximal Isometric Tetanus Stress*

345 The mean maximal isometric tetanus stress for EDL  $251 \pm 17$  kN/m<sup>2</sup> and diaphragm muscle  
346  $169 \pm 10$  kN/m<sup>2</sup> occurred at 10 weeks of age and is in keeping with values of 233-256 kN/m<sup>2</sup>  
347 for EDL [8, 31, 32] and 169kN/m<sup>2</sup> for diaphragm [6] from previous literature examining  
348 isometric stress from mice of a similar age group. Differences in strain and sex of mice, and  
349 environmental conditions in which they are kept prevent further comparison of age related  
350 results with accepted literature values. Absolute force values are provided in Table 1.

351 Tetanus stress was significantly affected by age (Fig 2B; PERMANOVA d.f. = 79; F =7.9; p  
352 =0.001). For both EDL and diaphragm muscle maximal isometric stress occurred at 10 weeks  
353 and was significantly lower at 3 (by 17% & 10% respectively), 30 (by 18% & 28%  
354 respectively), and 50 weeks of age (by 22% and 33% respectively; Fig 2B; pair-wise t > 2.1;  
355 p<0.05 in each case). In both cases, mean maximal tetanus stress was significantly reduced  
356 at 50 weeks compared to 3 weeks (Fig 2B; pair-wise t = 2.4; p =0.011).

#### 357 *Isometric Activation and Relaxation Times*



358 For both EDL and diaphragm muscle, mean time to half peak tetanus (THPT) and last  
359 stimulus to half relaxation (LSHR) were significantly affected by age (Fig 3; PERMANOVA d.f.  
360 = 79;  $F > 6.2$   $p = 0.001$  in both cases). THPT of 3 week EDL was significantly longer (by up to  
361 46%) than at 10, 30, and 50 weeks of age (Fig 3A; pair-wise  $t > 4.47$ ;  $p < 0.003$  in all cases).  
362 LSHR was significantly prolonged at 50 weeks of age (by up to 32%) compared to 3, 10 and  
363 30 weeks of age (Fig 3B; pair-wise  $t > 2.56$ ;  $p < 0.03$  in all cases).

364 In diaphragm muscle mean THPT was significantly longer (by 19%) at 30 weeks of age  
365 compared to at 10 weeks of age (Fig 3A; pair-wise  $t = 3.03$ ;  $p = 0.012$ ) and had a tendency to  
366 be greater than that at 3 weeks (Fig 3A; pair-wise  $t = 1.91$ ;  $p = 0.064$ ). LSHR was significantly  
367 greater at 50 weeks compared to 3 and 10 weeks (Fig 3B; pair-wise  $t > 1.9$   $p < 0.03$  in both  
368 cases).

#### 369 *Work Loop Power Output Normalised to Muscle Mass (Watts/kg)*

370 Work loop power output was significantly affected by age (Fig 4A; PERMANOVA d.f. = 79;  $F$   
371 = 4.6;  $p = 0.004$ ). For EDL mean maximal work loop power output peaked at 10 weeks of age  
372 and was significantly higher than at 3 (by 20%) and 50 weeks (by 13%: Fig 4A; pair-wise  $t > 2$ ;  
373  $p < 0.05$ ). In diaphragm mean maximal work loop power output was achieved at 10 weeks of  
374 age and was significantly reduced at 50 weeks (by 23%) (Fig 4A; pairwise  $t = 2.8$ ;  $p = 0.009$ ).  
375 Diaphragm work loop power output at 3 weeks of age was significantly greater than that at  
376 50 weeks (Fig 4A; pair-wise  $t = 2.61$ ;  $p = 0.024$ ).

#### 377 *Work Loop Power Output Normalised to Whole Animal Body Mass (Watts/g)*

378 Mean muscle PO, normalised to body mass, was significantly affected by age for EDL muscle  
379 (Fig 4B; PERMANOVA d.f. = 3, 36;  $F = 3.24$ ;  $p < 0.032$ ). For EDL mean maximal work loop  
380 power output, when normalised to body mass, was highest at 10 weeks of age and was

381 significantly reduced at 3 weeks (by 20%), 30 weeks (by 19%) and at 50 weeks of age (by  
382 22%) (Fig 4B; pair-wise  $t > 2.3$ ;  $p < 0.03$  in each case).

383 Similar calculations cannot be made for diaphragm muscle as whole diaphragm muscle mass  
384 was not measured.

#### 385 *Sustained Power Output*

386 Muscle power output during repetitive work loop activation was significantly affected by age  
387 in both EDL and diaphragm muscle (Fig 5A; PERMANOVA d.f. = 3, 36;  $F > 6.3$ ;  $p < 0.002$  in  
388 both cases). For EDL, the ability to sustain muscle power output over time was significantly  
389 reduced at 50 weeks compared to all other age groups (Fig 5; pair-wise  $t > 2.8$ ;  $p < 0.001$  in  
390 both cases). Similarly sustained muscle power output of 10-week-old EDL was significantly  
391 reduced compared to 3 and 30 weeks of age (Fig 5A; pair-wise  $t > 2.4$ ;  $p < 0.02$  in each cases).

392 Sustained muscle power output of 10 week old diaphragm muscle was significantly reduced  
393 compared to that at 3 weeks (Fig 5B: pair-wise  $t = 4.72$ ;  $p < 0.001$ ) and had a tendency to be  
394 lower than at 30 weeks (Fig 5B; pair-wise  $t = 2$ ;  $p = 0.0621$ ). Furthermore sustained work loop  
395 power output in 50-week-old diaphragm was significantly lower than that at 3 weeks (Fig 5B;  
396 pair-wise  $t = 3.84$ ;  $p = 0.002$ ). There was a tendency for sustained muscle power output at 30  
397 weeks to be lower than that at 3 weeks (Fig 5B; pair-wise  $t = 1.74$ ;  $p = 0.098$ ), but beyond this  
398 no other significant differences were found (Fig 5B; pair-wise  $t > 0.98$ ;  $p > 0.15$  in all cases).

399 Typical work loop shapes indicate (Figures 6 & 7) that in muscles where the reduction in  
400 power output occurred more rapidly (10, 50 week EDL & 10 week diaphragm) there was an  
401 increased relaxation time during re-lengthening phase over the course of the protocol,  
402 resulting in greater negative work and further contributing to the loss of net work (positive  
403 work during shorting – negative work during muscle re-lengthening) through repetitive  
404 activation.

405                    *Recovery from Sustained Work loop Activation*

406    There was a significant effect of age on the recovery of muscle power output post repetitive  
407    work loop activation in EDL muscle (Fig 8A; PERMANOVA d.f. = 3, 32;  $F = 10.2$ ;  $p < 0.001$ ).

408    Mean recovery of EDL at 3 weeks of age was significantly greater than at 10, 30 and 50  
409    weeks of age (Fig 8A; pair-wise  $t > 2.51$ ;  $p < 0.007$  in all cases). Recovery at 30 weeks of age  
410    was significantly reduced compared to 10 and 50 weeks of age (Fig 8A; pair-wise  $t > 3.24$ ;  $p$   
411     $< 0.006$ ).

412    Peak recovery of diaphragm muscle did not differ between age groups (Fig 8B; PERMANOVA  
413    d.f. = 3, 35;  $F = 0.33$ ;  $p = 0.978$ ).

414                    *Biochemical Analysis*

415    In EDL muscle SERCA, CS and LDH activity were significantly affected by age (Fig 9A;B;C; d.f. =  
416    3, 26  $F > 3.11$ ;  $p < 0.03$  in each case). SERCA at 50 weeks was significantly lower than at 10  
417    and 30 weeks (9A pair-wise  $t = 2.36$ ;  $p < 0.02$  in both cases), and had a tendency to be lower  
418    than at 3 weeks of age (9A; pair-wise  $t = 2.72$ ;  $p < 0.008$ ). CS activity was significantly lower at  
419    10 weeks compared to all other ages (9B; pair-wise  $t > 4$ ;  $p < 0.003$  in all cases). CS activity at  
420    3 weeks was significantly lower than that at 30 and 50 weeks of age (9B; pair-wise  $t > 4$ ;  
421     $p < 0.004$  in both cases). LDH activity of 3-week old mice was significantly lower than at 10  
422    and 50 weeks (Figure 9C; pair-wise  $t > 3.75$ ;  $p < 0.005$  in both cases) and had a tendency to be  
423    lower than at 30 weeks of age (Figure 9C; pair-wise  $t = 2.03$ ;  $p = 0.058$ ).

424    For diaphragm muscle LDH activity changed significantly with age (Fig: 10C; PERMANOVA d.f.  
425    = 3, 32 respectively;  $F = 3.42$ ;  $p = 0.02$ ). LDH activity was significantly lower at 3 weeks than at  
426    10 and 30 weeks (Figure 9C; pair-wise  $t > 2.28$ ;  $p < 0.02$  and had a tendency to be lower that  
427    at 50 weeks of age (Figure 9C;  $t = 1.8$ ; pair-wise  $p = 0.069$ ). There were no significant  
428    differences in SERCA or CS activity (9A;B; PERMANOVA d.f. = 3, 28;  $F < 1.6$ ;  $p > 0.2$ ).

429 mRNA for SERCA1, SERCA2, RYR1, Tnni1, Tnni2 was not significantly different between age  
430 groups in either EDL or diaphragm (Fig 10; PERMANOVA d.f. = 3, 20 & 3, 22 for EDL and  
431 diaphragm measures respectively;  $F > 0.72$ ;  $p > 0.01$  in all cases).

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449 **Discussion**

450 The present work is the first to use the work loop technique as a better estimate of *in vivo*  
451 muscle power production to demonstrate an age and muscle specific decline in maximal  
452 mechanical function of isolated mammalian skeletal muscle that starts to occur at a  
453 relatively young age. More significantly the limited change in the examined biochemical  
454 parameters suggest that the age related reduction in performance occurs with only minor  
455 changes in muscle metabolic capacity, and in the case of EDL, without prevalent atrophy.

456 *Effect of Age on Maximal Skeletal Muscle Force, Power Output, and Activation and*  
457 *Relaxation Times*

458 EDL and diaphragm muscle from 10-week-old mice produced the highest isometric stress,  
459 lowest activation and relaxation times, highest power output and appeared to have a faster  
460 fibre type composition. In contrast to EDL, these parameters were already well developed in  
461 3 week old diaphragm, and subsequently may underline the importance of the physiological  
462 function of breathing in comparison to locomotory performance in the early stages of life.  
463 The reported differences between the tested muscles are likely to relate to the speed of  
464 development of the contractile properties during growth between 3 and 10 weeks of age.  
465 Skeletal muscle maximal force and the rate of force development are largely related to the  
466 efficiency of the excitation contraction coupling process, and more specifically, the rate and  
467 quantity of SR Ca<sup>2+</sup> release into the intramuscular space [13]. At birth muscular SR is a loose  
468 network of tubes limited in quantity, and has been demonstrated to increase in a fibre  
469 specific manner [44, 60]. Previous findings have suggested the SR content of skeletal muscle  
470 with a predominantly faster phenotype at maturity takes longer to develop, and as such, the  
471 optimised process excitation contraction coupling occurs at a later age [44]. This coincides  
472 with a prolonged time in the development of faster muscle fibres during growth, and  
473 previous research by Agbulut *et al.* [1] demonstrated that 21 days post gestation type IIb

474 myosin heavy chain represented 54% of the total proportion of EDL, which increased to 87%  
475 at 90 days. As is widely recognised, these fibres coincide with a greater normalised maximal  
476 force and power output and more rapid activation time due to enhanced contractile  
477 characteristics and glycolytic potential [64, 74]. In support of this the reduction in LDH and  
478 increase in CS in the present study indicates a greater oxidative capacity in 3 week EDL than  
479 was found in 10 week EDL.

480 Increasing age beyond 10 weeks was associated with a reduction in maximal isometric stress  
481 and work loop power, which was more greatly pronounced in diaphragm muscle.  
482 Contradictory to research suggesting that the loss of muscle strength is greater in magnitude  
483 than the loss of power [20, 48, 62], the present findings infer that the reduction in isometric  
484 muscle stress was 10% greater than the loss of work loop power. Furthermore a reduction in  
485 isometric stress was seen as early as 30 weeks of age whilst maximal power output was  
486 maintained until 50 weeks of age.

487 The current work extends the finding by Chan and Head [16], which concluded that a  
488 significant reduction in the tetanic stress of 20-22 month old female mice occurred without  
489 prevalent atrophy, by uniquely demonstrating an age related decline in dynamic muscle  
490 power that occurs in the absence of muscle atrophy and at a much younger age. Conversely,  
491 the muscle mass of EDL in the present study was significantly increased at 50 weeks and  
492 probably relates to a greater morphological size of the animal. Although evidence suggests  
493 that muscle mass is lost with ageing, the extent of such loss is variable and muscle group  
494 specific [14, 15, 55]. Brown and Hasser [15] suggested that this controversy may arise due to  
495 differences in the strain of rodents examined, the use of non-pathogen free animals and the  
496 age of the animals deemed to be aged. It has been suggested that significant muscle atrophy  
497 takes place in the final 20% of the animal's lifespan [16] and subsequently the ages of mice

498 in the present study precede this. The current findings indicate that muscular atrophy is not  
499 the sole contributor to reduced muscle performance during early ageing.

500 A primary mechanism for the decline in mechanical performance in older age groups,  
501 appears to be a shift towards a slower more oxidative fiber type [5, 7, 17], which  
502 subsequently results in a reduced potential to produce high force. Despite this, research  
503 suggests that older ageing evokes a reduction in oxidative capacity of the muscle largely  
504 attributed to a decline in mitochondrial function [17, 61], which is characterised by a  
505 reduction in oxidative enzymes such as CS [61]. Interestingly, the given increase in CS in 50-  
506 week-old EDL muscle in the present study contradicts this, and it may therefore be  
507 considered that an early age related shift to slower fibre type may be effective in offsetting  
508 the decline in mitochondrial function, due to the enhanced oxidative capacity of such  
509 phenotypes. Furthermore, as there were no concomitant changes in biochemical parameters  
510 of 50 week old diaphragm, this indicates that the early age related reduction in mechanical  
511 performance may in part relate to mechanisms other than a change in muscle metabolic  
512 capacity.

513 The age related reduction in muscular contractility may therefore relate to an increase in  
514 dysfunctional  $\text{Ca}^{2+}$  handling proteins. The most documented of which is the uncoupling of  
515 DHPR-ryanodine receptors resulting in a reduced  $\text{Ca}^{2+}$  availability at the contractile proteins  
516 [19, 40, 53, 58]. Furthermore, the present findings support previous research indicating an  
517 age induced inactivation of SERCA [40, 68]. Interestingly, the reduction in SERCA activity  
518 does not correspond with a reduction in mRNA transcript content, which suggests the build-  
519 up of dysfunctional SERCA proteins, rather than a loss in number, is more prevalent during  
520 early ageing. The reported age related reduction in SERCA activity corresponds to the  
521 increase in relaxation time seen in EDL muscle in the present study [29, 40, 52].

522 When normalised to animal body mass, the reduction in muscle power output ( $W \cdot g^{-1}$ ) from  
523 10 week to 50 week EDL, of approximately 22%, was equal in magnitude to the loss of  
524 maximal force. Therefore, the animal is likely to move at a reduced pace and fatigue more  
525 quickly at the same relative intensity.

#### 526 *Effect of Age on Sustained Muscle Power Output*

527 The present results infer an age and muscle specific ability to maintain power output during  
528 repetitive stimulation, although a typical pattern was established. 3-week-old muscle  
529 demonstrated the greatest ability to sustain power output which significantly reduced at 10  
530 weeks. Following this sustained power output was significantly greater at 30 weeks before a  
531 second wave of reduced sustained muscle power occurred at 50 weeks. The relative  
532 magnitude of these changes was muscle specific and this diverse and complex spectrum of  
533 findings is likely affected by growth, development and age; such complex changes over an  
534 animals lifespan likely gives rise to the equivocal *in vivo* and *in vitro* results that have  
535 previously examined the effect of ageing on muscular endurance [9, 11, 18, 30, 36, 41, 43,  
536 55].

537 In relation to previous findings on muscle fibre type composition development during  
538 growth [1], the enhanced ability to maintain muscle power output over repetitive  
539 stimulation in 3 week old muscle is likely to relate to a slower phenotype and an increased  
540 oxidative capacity as indicated by the reduced LDH activity in both diaphragm and EDL  
541 muscle and further elevated CS in EDL. Although the similarities in mechanical performance  
542 between 3 and 10 week old diaphragm may appear to contradict this, Agbulut et al. [1]  
543 indicate that the increased number of neonatal fibres may be compensated by an increased  
544 type IIb fibre expression.



545 Previous isolated muscle research demonstrating increased [16, 55], decreased [73] and  
546 negligible [24] effects in the maintenance of muscle force with increasing age via repetitive  
547 isometric contractions, are difficult to compare to the findings in the present study due to  
548 potential differences in the fatigue mechanism promoted by the work loop technique. Any  
549 age related changes in muscle activation and relaxation time, ability of the muscle to  
550 maintain force through shortening, maximal shortening velocity and passive resistance to  
551 stretch will have profound additional effects on the muscle ability to sustain power output in  
552 work loops to any changes in ability to produce force.

553 The age related decline in muscle stress and ability to maintain power observed in 50 week  
554 old muscle may further relate to an age induced increase in muscle collagen and fat resulting  
555 in larger non-contractile mass and subsequent muscle stiffness [5, 36, 46]. This increased  
556 resistance to stretch would amplify the proportion of negative work and decrease the  
557 maximal net work loop power output (work loop power output = positive work – negative  
558 work: [35]).

559 Unlike diaphragm muscle, 50 week EDL had the poorest ability to sustain power. This may be  
560 in part attributed to a more greatly pronounced increase in eccentric work during the re-  
561 lengthening phase of the work loop, as indicated by the work loop shapes. If the muscle is  
562 active during re-lengthening, a greater proportion of negative work is conducted and thus  
563 the net work production per cycle is significantly reduced. Irrespective of ageing, fatigue is  
564 associated with an increase in relaxation time in successive work loops [2, 9, 65];  
565 accumulation of this effect combined with the demonstrated age related increase in  
566 relaxation time in the present study is likely to result in a greater reduction in power output  
567 from older animals, particularly in EDL muscle.

568 *Effect of Age on Recovery from Repetitive Stimulation*

569 The recovery of diaphragm muscle was not affected by age. Conversely, EDL muscle from 3-  
570 week-old mice recovered to the greatest degree and recovery at 30 weeks of age was  
571 significantly reduced.

572 Although the acute response of the contractile properties following muscular fatigue in the  
573 aged population has received little attention, particularly in isolated muscle, human and  
574 animal evidence suggests that recovery is largely unaffected [4, 23]. Gonzalez and Delbono  
575 [22] concluded that despite changes in maximal tetanic stress of EDL and soleus muscle from  
576 22-24 month old mice, recovery time and stress production following fatigue via repetitive  
577 isometric contractions were unaffected by age.

578 Previous findings using the work loop technique have demonstrated that the recovery of  
579 power output occurs faster in muscle with a slower fibre type [65]. Consequently this may  
580 explain why EDL muscle from 3-week-old mice recovered more quickly than EDL at other  
581 ages in the present study, and why diaphragm muscle recovered much more quickly than  
582 EDL muscle. There is no plateau in the recovery of EDL muscle during this period and it is  
583 likely that given a longer duration, this increase in muscle power would continue up to 60  
584 minutes to approximately 80-90% as demonstrated in our previous work [32, 28].

#### 585 *Limitations & Practical Implications of the Study*

586 The present research is conducted using female mice and, although the overall trends  
587 demonstrated in the present study are unlikely to change, the time course and magnitude of  
588 the ageing response is likely to differ in male mice due to sex related differences in hormone  
589 secretion [16, 49]. Although there is some evidence in female mice [49], previous studies  
590 examining the effect of ageing on the contractile properties of isolated rodent skeletal  
591 muscle have largely focused on males [14, 26, 45, 66]. To the author's knowledge, research  
592 by Chan and Head [16], is the only study to assess the age and sex related changes in skeletal

593 muscle contractility. Chan and Head [16] demonstrated that the age related decline in  
594 maximal absolute force and increase in isometric relaxation time of EDL from 22 month old  
595 mice appeared to affect females to a greater extent; however there were no sex related  
596 difference in the decline in maximal specific force. With the previously examined effect of  
597 increased relaxation time on work loop power and the muscle specific ageing response  
598 discussed in the present study, future investigation should further examine the age and sex  
599 related decline in skeletal muscle contractility.

600 As previously suggested, ageing may promote a greater non-contractile mass, and as such  
601 the  $1060 \text{ kg m}^{-3}$  value used in our calculations may overestimate muscle density in older  
602 animals, and as a result underestimate CSA, in muscles from the older age groups. This may  
603 result in stress being over estimated in older muscles and it is therefore considered that the  
604 reduction in maximal stress may be greater than that portrayed in the present study. In  
605 addition to this we recognise that previous studies examining the mechanical function of  
606 EDL have used slight variations in calculation of estimated mean muscle fibre length, which  
607 will affect the calculation of maximal stress. Although the calculation used in the present  
608 study has been used in previous work [28, 31, 32], absolute isometric force data has been  
609 included (Table 1) to allow further comparison of maximal isometric force across the  
610 literature. Importantly, a change in the calculation of EDL fibre length will not affect the  
611 demonstrated trend and magnitude of effect shown in presented results.

612 Having an improved understanding of the ageing response is important in the potential  
613 development of innovations to improve human health and quality of life [20]. The present  
614 study highlights significant reductions in skeletal muscle performance that occur at a  
615 relatively young age, and such effects are likely to be magnified in older age groups. Early  
616 ageing was associated with a greater loss of diaphragm force and power compared to  
617 locomotory EDL muscle which may warrant further research investigating the contribution

618 of diaphragm muscle to the severity of respiratory symptoms observed in elderly patients  
619 [54, 56]. Furthermore, the suggested age related increase in central fatigue that occurs in  
620 endurance tasks may potentially magnify the ageing response seen in the present study  
621 when relating these results to *in vivo* performance [13, 20].

622 It was interesting to note that, although not statistically significant, higher body mass  
623 resulted in a 25% decrease in power output in 50 week old diaphragm. Skeletal muscle lipid  
624 accumulation has been demonstrated to have a negative impact on the maintenance and  
625 regeneration of contractile proteins [2], and is believed to further cause insulin resistance,  
626 with diabetes being associated with reduced skeletal muscle metabolic capacity [27]. The  
627 direct effect lipid accumulation on skeletal muscle mechanical performance has not yet been  
628 studied and would be an interesting area of future research.

#### 629 *Conclusion*

630 The present findings indicate that the loss of skeletal muscle mechanical function is  
631 significant at a relatively young age and more profound in diaphragm. Our findings indicate  
632 that this reduction in muscle performance occurs without prevalent atrophy mechanisms,  
633 and with potentially limited change in fibre type. In contrast to previous human research,  
634 the reduction in maximal muscular force exceeded the loss in maximal power, which may  
635 indicate that a loss in power is a consequence of the further interaction between muscle  
636 atrophy and deterioration in neuromuscular innervation. Furthermore, the present findings  
637 show an age and muscle specific ability to sustain muscle power output over repetitive  
638 activation, which helps to rationalise previous equivocal findings examining the effect of  
639 ageing on muscular fatigue.

#### 640 *Perspectives and Significance*

641 The evidence presented in the present study is the first to offer a muscle specific insight into  
642 the early ageing effect on skeletal muscle contractility using methods that more accurately  
643 represent muscle action *in vivo*. The present study highlights significant reductions in  
644 skeletal muscle performance that occur at a relatively young age. Having an improved  
645 understanding of the ageing response is important in the potential development of  
646 innovations to improve human health and quality of life. The future direction of this  
647 research area should be to investigate the contribution of obesity and a sedentary lifestyle  
648 to the muscle ageing response.

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Table 1. Mean absolute twitch and tetanus force and muscle mass for EDL and diaphragm muscle at each age

Muscle		Age (Weeks)			
		3	10	30	50
EDL	Twitch Force (mN)	42±3.9	100.3±3.3	85.9±5.1	94.5±8
	Tetanus Force (mN)	193.9±14.3	337.3±20.1	300±11.4	335.6±21.2
	Muscle mass (mg)	6.72±0.5	12.44±0.4	14.2±0.2	15.99±0.8
Diaphragm	Twitch Force (mN)	46.9±3.9	76.6±6	74.6±5.9	88.1±9.2
	Tetanus Force (mN)	192.5±11	295.9±21.8	319.2±24.7	314.4±23.8
	Muscle mass (mg)	7.71±0.6	15.48±0.7	21.05±6.7	27.35±1.6

888 Data represented as mean±s.e.m: n = 10 for each group. Data for EDL represents whole

889 muscle mass and for diaphragm the mass represents the section of the muscle used in the

890 evaluation of mechanical performance.

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908 **Figure Captions**

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910 Figure 1. – Increasing age resulted in greater mean body mass of CD1 mice (**A**) and higher  
911 EDL muscle mass (**B**). A subdivision in the 50 week data is highlighted by **X&Y**. Data labelled  
912 **X** represent 50 week old mice with body mass greater than 70g, whereas data labelled **Y**  
913 represents 50 week old mice with body mass below 50g [Data represented as mean  $\pm$  s.e.m;  
914 n=20 for each age group (**A**); n=10 for each group (**B**); significant differences between age  
915 groups are indicated by them having common symbols]

916

917 Figure 2. – The effect of age on mean maximal isometric twitch and tetanus stress in mouse  
918 EDL (A & C) and diaphragm (B & D) muscle. Increasing age, from maturity, generally caused  
919 a decrease in maximal isometric twitch and tetanus stress in EDL and diaphragm muscle.  
920 Maximal twitch and tetanus stress were significantly lower in the oldest age group tested  
921 when compared to the peak stress achieved at 10 weeks of age. [Data represented as mean  
922  $\pm$  s.e.m: n=10 in each case; significant differences between age groups are indicated by them  
923 having common symbols]

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925 Figure 3 – The effect of age on mean isometric tetanus muscle activation time ( THPT; time  
926 to half peak tetanus) and relaxation time (LSHR; last stimulus to half tetanus relaxation) in  
927 mouse EDL (A & C) and diaphragm muscle (B & D). THPT was significantly longer in 3 week  
928 old EDL, but beyond this there was little change in THPT with increasing age in both EDL and  
929 diaphragm muscle. LSHR was significantly longer at 50 weeks, than at 10 weeks, in both EDL  
930 and diaphragm [Data represented as mean  $\pm$  s.e.m: n=10 in each case; significant differences  
931 between age groups are indicated by them having common symbols]

932 Figure 4. - The effect of age on mean maximal work loop power output plotted as Watts per  
933 kilogram muscle mass for mouse EDL (A) and diaphragm (B) muscles and Watts per gram  
934 body mass for EDL (C). Maximal power output was achieved at 10 weeks of age in both EDL  
935 and diaphragm muscle and beyond this, increasing age was associated with a significant  
936 reduction in muscle power output. [Data represented as mean  $\pm$  s.e.m: n=10 in each case;  
937 significant differences between age groups are indicated by them having common symbols]

938

939 Figure 5. - The effect of age on sustained muscle power output during repetitive work loop  
940 activation in mouse EDL and diaphragm muscle. The ability to maintain power through  
941 repetitive activation was muscle specific, however there was a general pattern of age related  
942 changes with greatest maintenance of power at 3 weeks, reduced at 10 weeks, increased at  
943 30 weeks, then reduced again at 50 weeks in both EDL and diaphragm muscles. [Data  
944 represented as mean  $\pm$  s.e.m: n=10 in each case; wks = weeks of age; significant differences  
945 between age groups are indicated by them having common symbols]

946

947 Figure 6. - The effect of age on typical work loop shapes of mouse EDL muscle during  
948 repetitive activation at 10Hz cycle frequency for 3 week old mice, 10 week old mice, 30 week  
949 old mice and 50 week old mice. The figures depict work loops 2 (0.2s of the protocol), 10 (1s)  
950 and 18 (1.8s) of the fatigue run. The eccentric muscle activity in the re-lengthening phase of  
951 the work loop was increased in fatigued muscles from 10 week to 50 week old EDL. EDL  
952 muscles from this oldest age group were associated with the poorest fatigue resistance.

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954 Figure 7. - The effect of age on typical work loop shapes of mouse diaphragm muscle during  
955 repetitive activation at 7Hz cycle frequency for 3 week old mice, 10 week old mice, 30 week  
956 old mice and 50 week old mice. The figures depict work loops 2 (0.29s of the protocol), 10

957 (1.43s) and 18 (2.57s) of the fatigue run. The eccentric muscle activity in the re-lengthening  
958 phase of the work loop was increased in fatigued muscles from 10 week old diaphragm (B)  
959 when compared with other age groups. Diaphragm muscles from this age group were  
960 associated with the poorest fatigue resistance.

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962 Figure 8 - The effect of age on mean recovery of power output of mouse EDL (A) and  
963 diaphragm (B) muscle following a protocol a repetitive work loop activity. There was an  
964 increase in muscle power output, over time, in EDL muscle with significantly greater  
965 recovery in 3 week EDL compared to all other age groups. Peak recovery of diaphragm  
966 muscle occurred after 10 minutes but there were no significant differences in the recovery  
967 pattern between age groups. [Data represented as mean  $\pm$  SE: n=10 for 10 & 30 weeks; n=9  
968 for 50 weeks; n=8 for 3 weeks; wks = weeks of age; significant differences between age  
969 groups are indicated by them having common symbols]

970

971 Figure 9 – The effect of age on EDL and diaphragm muscle activities of SERCA, CS and LDH  
972 SERCA was significantly decreased and CS and LDH significantly increased in 50 week EDL  
973 muscle. Diaphragm LDH activity was higher at 10 weeks when compared to that from 3  
974 weeks old mice, but beyond this there were limited changes in the measured enzyme  
975 activities. [Data represented as mean  $\pm$  s.e.m: n=8-10 in each case; significant differences  
976 between age groups are indicated by them having common symbols]

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978 Figure 10 – There was no significant effect of increasing age on relative mRNA  
979 concentrations of SERCA1, SERCA2, RYR1, Tnni1, and Tnni2 in EDL and diaphragm muscle  
980 quantified by qRT-PCR [Data normalised to 3 week old mice and represented as mean  $\pm$   
981 s.e.m: n=8-10 in each case]

Figures

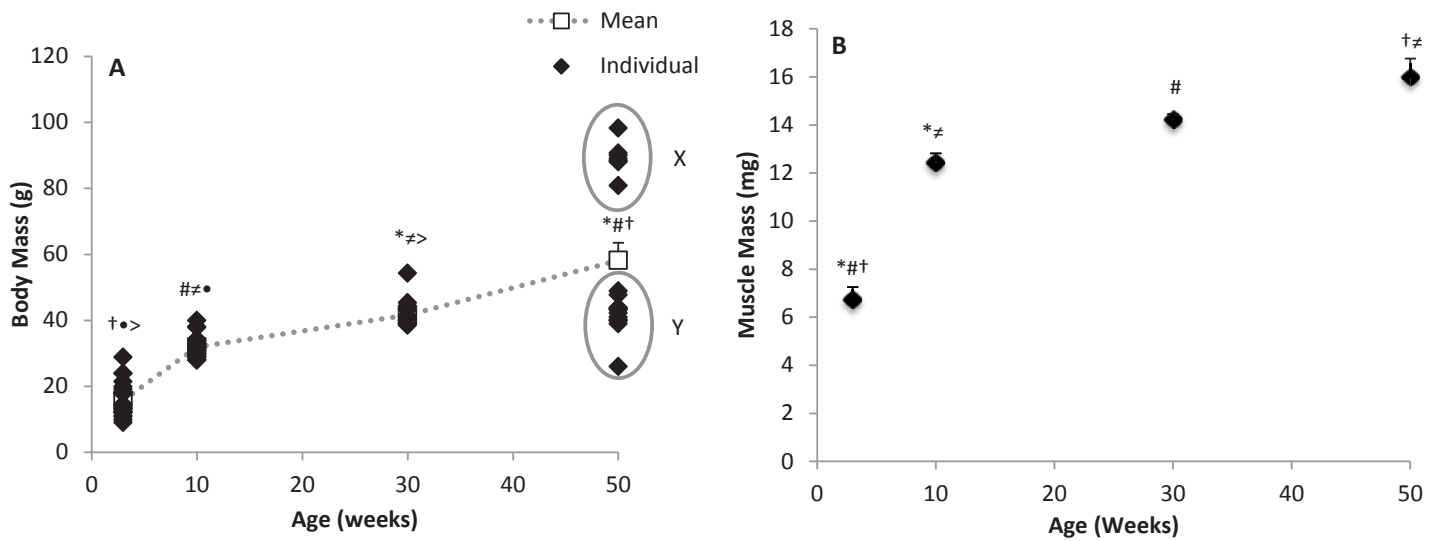


Figure 1. – Increasing age resulted in greater mean body mass of CD1 mice (A) and higher EDL muscle mass (B). A subdivision in the 50 week data is highlighted by X&Y. Data labelled X represent 50 week old mice with body mass greater than 70g, whereas data labelled Y represents 50 week old mice with body mass below 50g [Data represented as mean  $\pm$  s.e.m; n=20 for each age group (A); n=10 for each group (B); significant differences between age groups are indicated by them having common symbols]



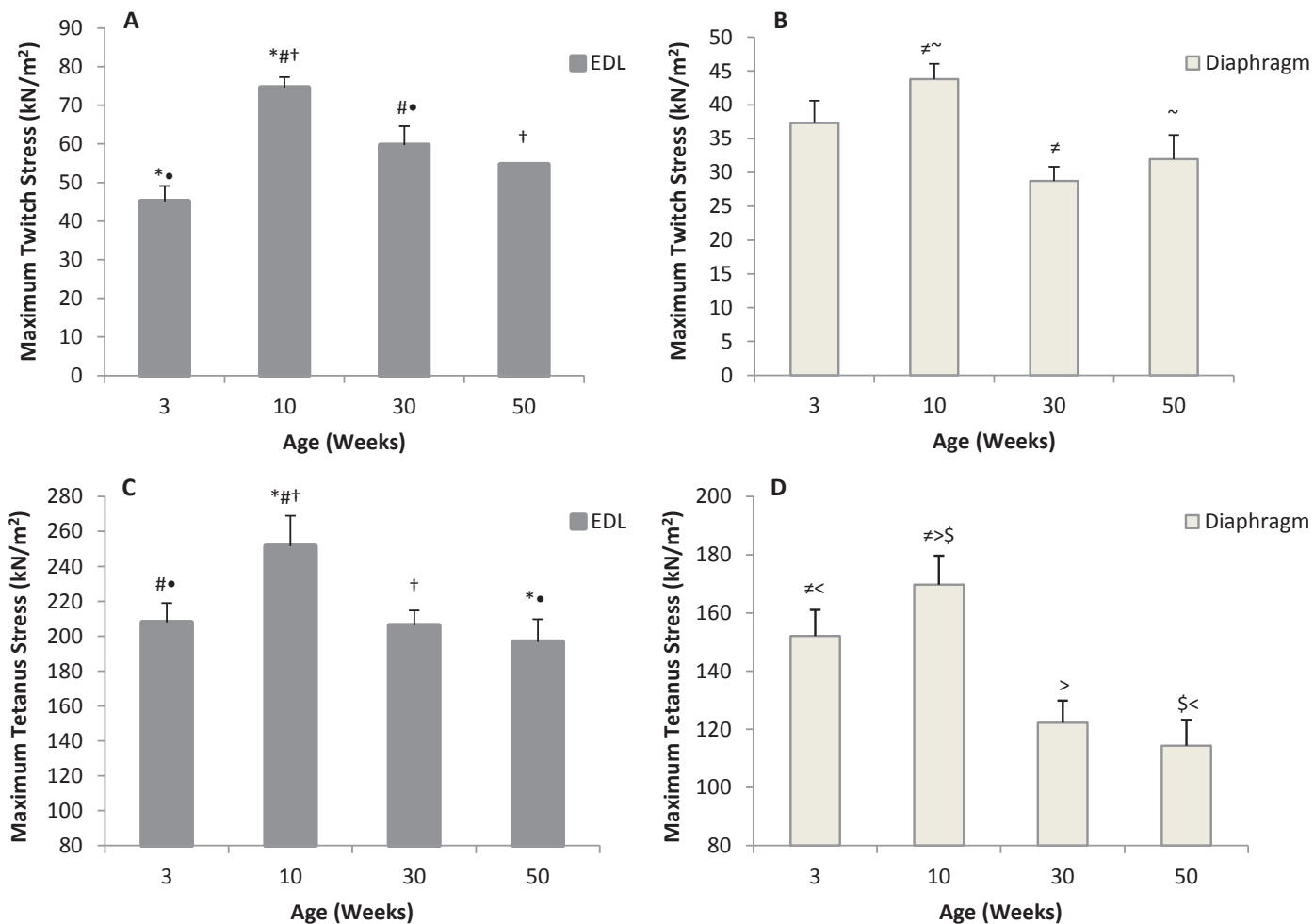


Figure 2. – The effect of age on mean maximal isometric twitch and tetanus stress in mouse EDL (A & C) and diaphragm (B & D) muscle. Increasing age, from maturity, generally caused a decrease in maximal isometric twitch and tetanus stress in EDL and diaphragm muscle. Maximal twitch and tetanus stress were significantly lower in the oldest age group tested when compared to the peak stress achieved at 10 weeks of age. [Data represented as mean  $\pm$  s.e.m: n=10 in each case; significant differences between age groups are indicated by them having common symbols]

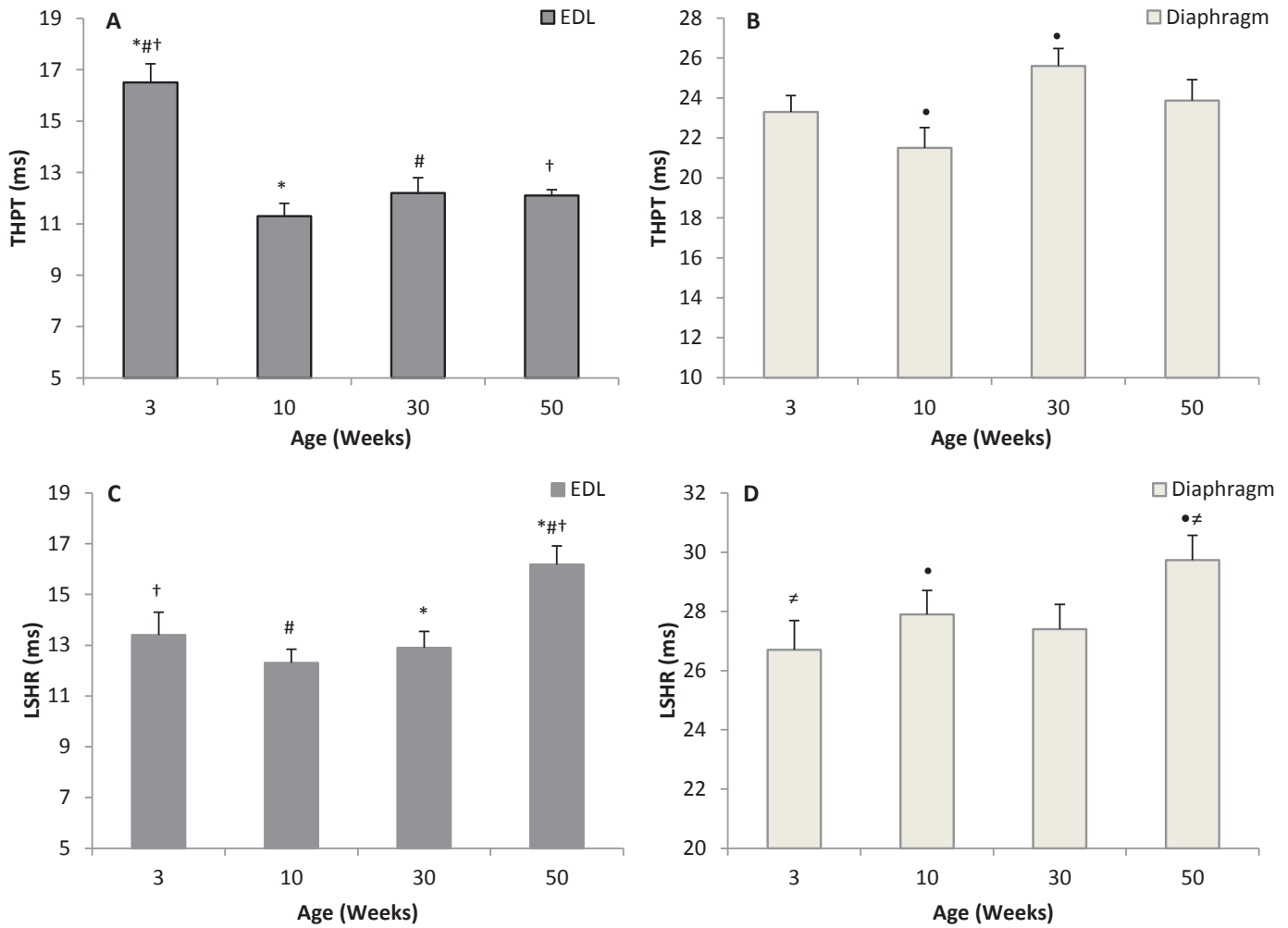


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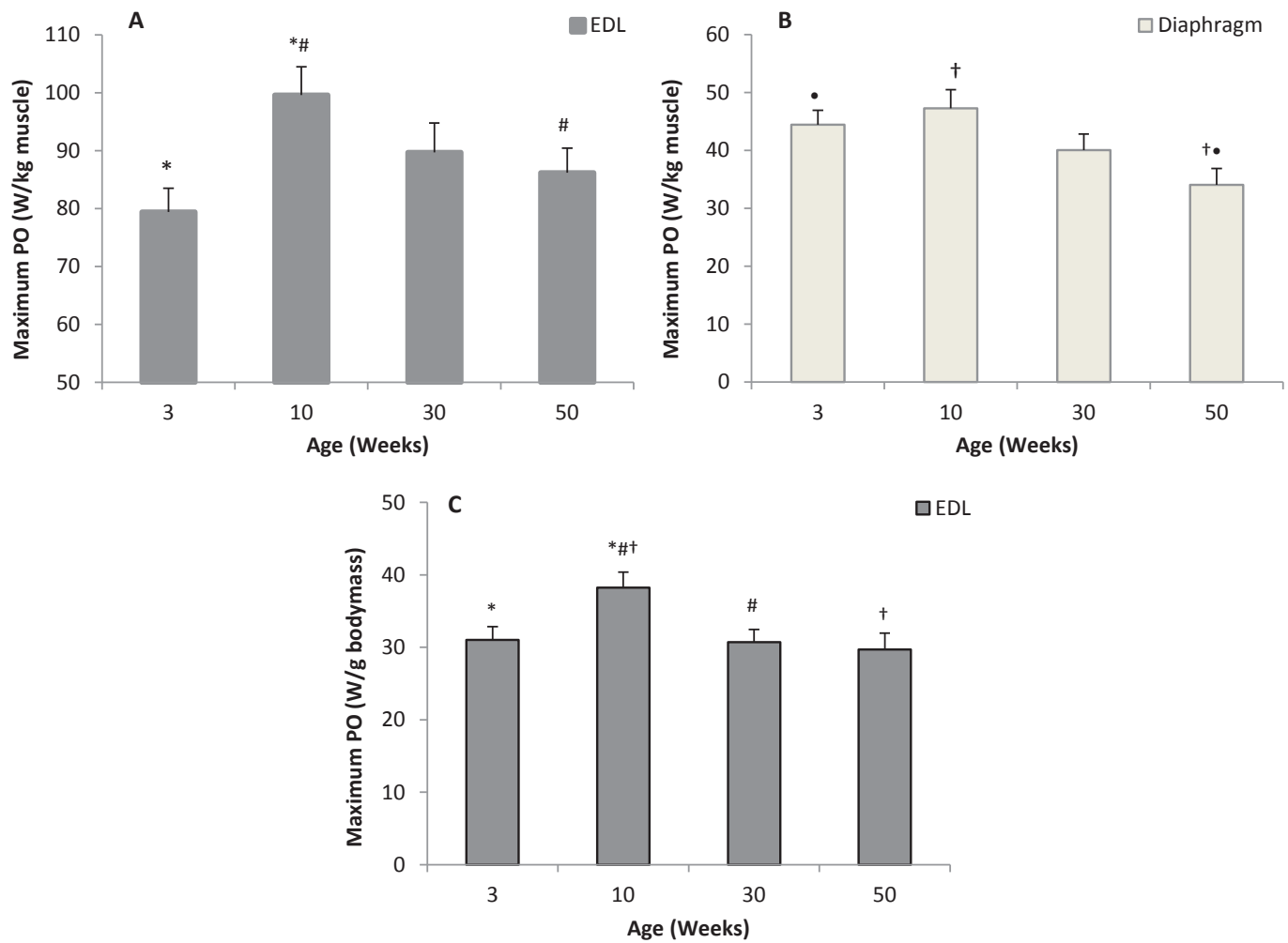


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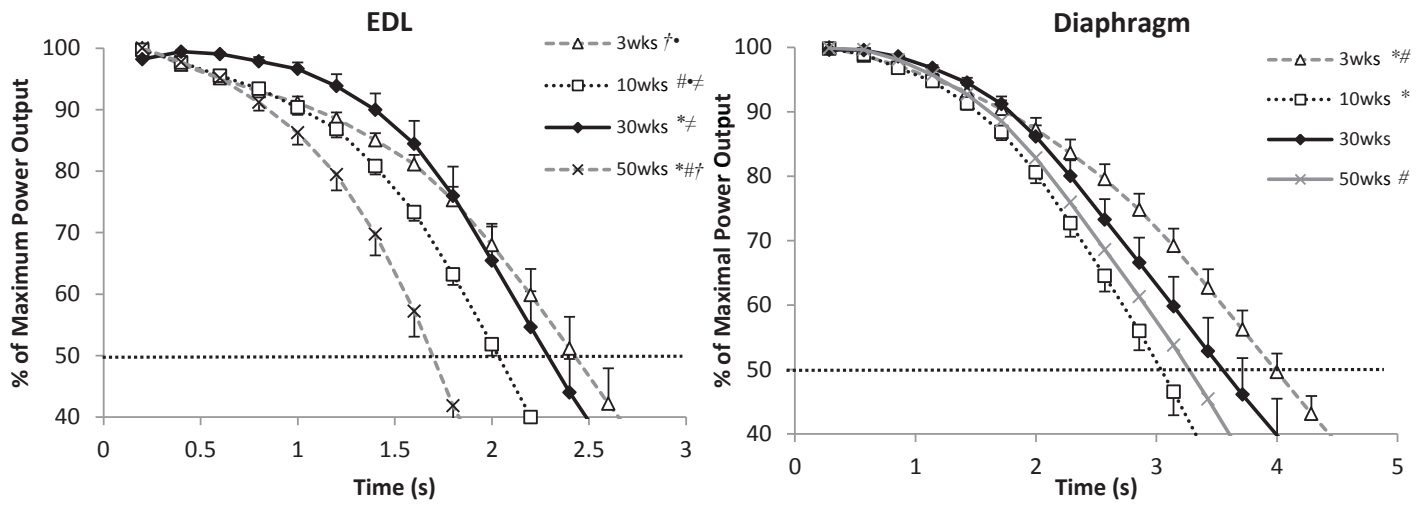


Figure 5. - The effect of age on sustained muscle power output during repetitive work loop activation in mouse EDL and diaphragm muscle. The ability to maintain power through repetitive activation was muscle specific, however there was a general pattern of age related changes with greatest maintenance of power at 3 weeks, reduced at 10 weeks, increased at 30 weeks, then reduced again at 50 weeks in both EDL and diaphragm muscles. [Data represented as mean  $\pm$  s.e.m: n=10 in each case; wks = weeks of age; significant differences between age groups are indicated by them having common symbols]

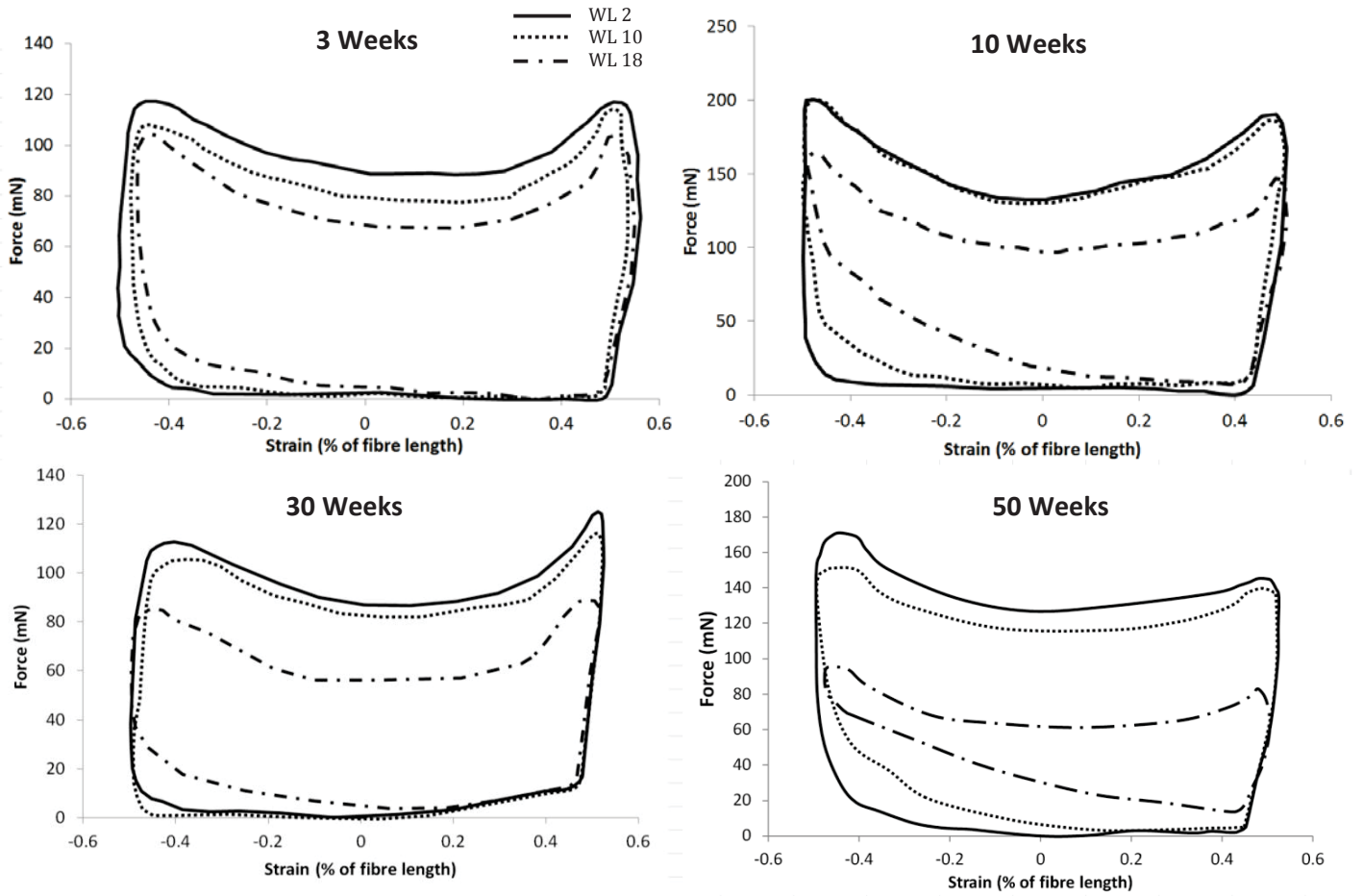


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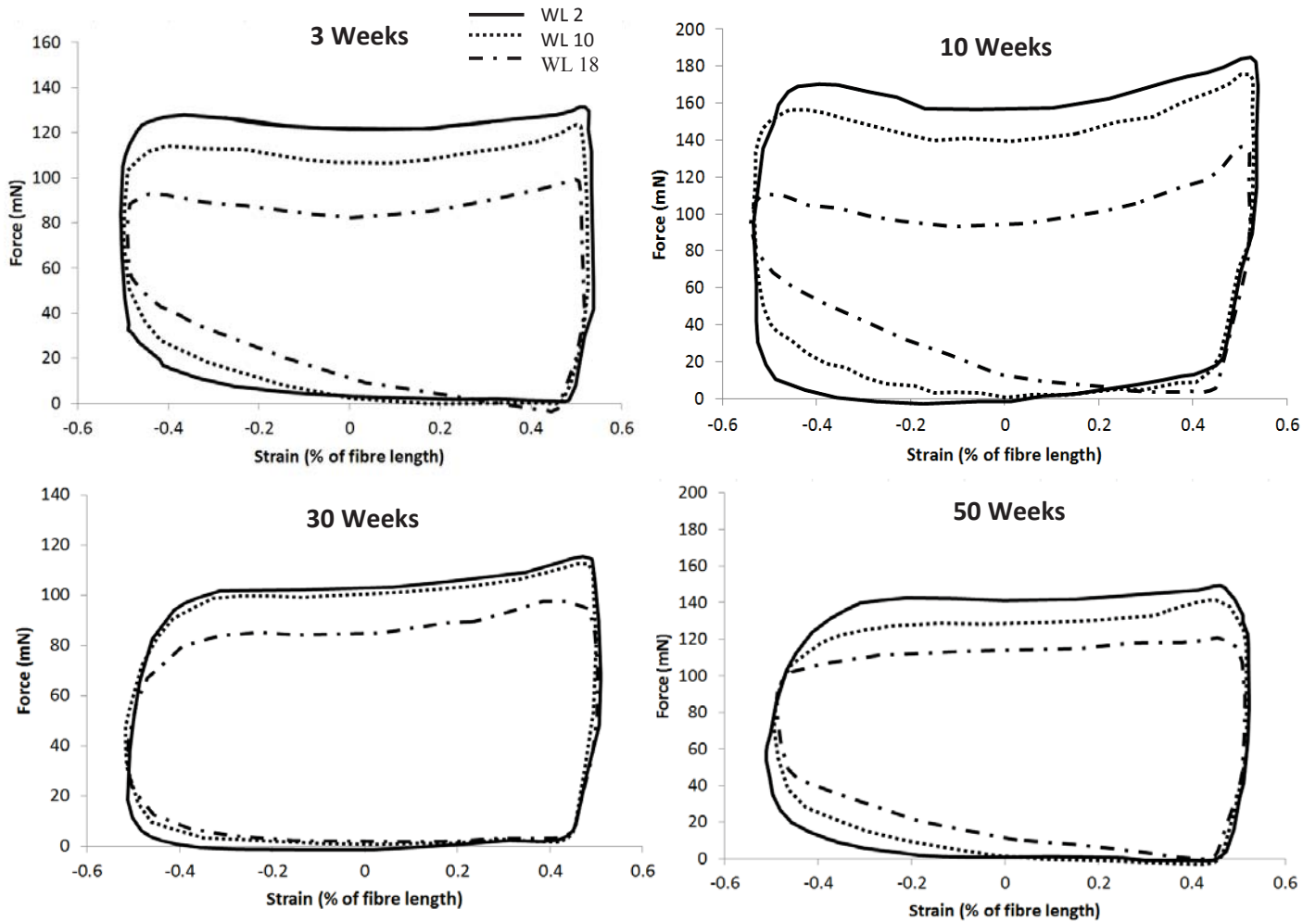


Figure 7. - The effect of age on typical work loop shapes of mouse diaphragm muscle during repetitive activation at 7H cycle frequency for 3 week old mice, 10 week old mice, 30 week old mice and 50 week old mice. The figures depict work loops 2 (0.29s of the protocol), 10 (1.43s) and 18 (2.57s) of the fatigue run. The eccentric muscle activity in the re-lengthening phase of the work loop was increased in fatigued muscles from 10 week old diaphragm (B) when compared with other age groups. Diaphragm muscles from this age group were associated with the poorest fatigue resistance.

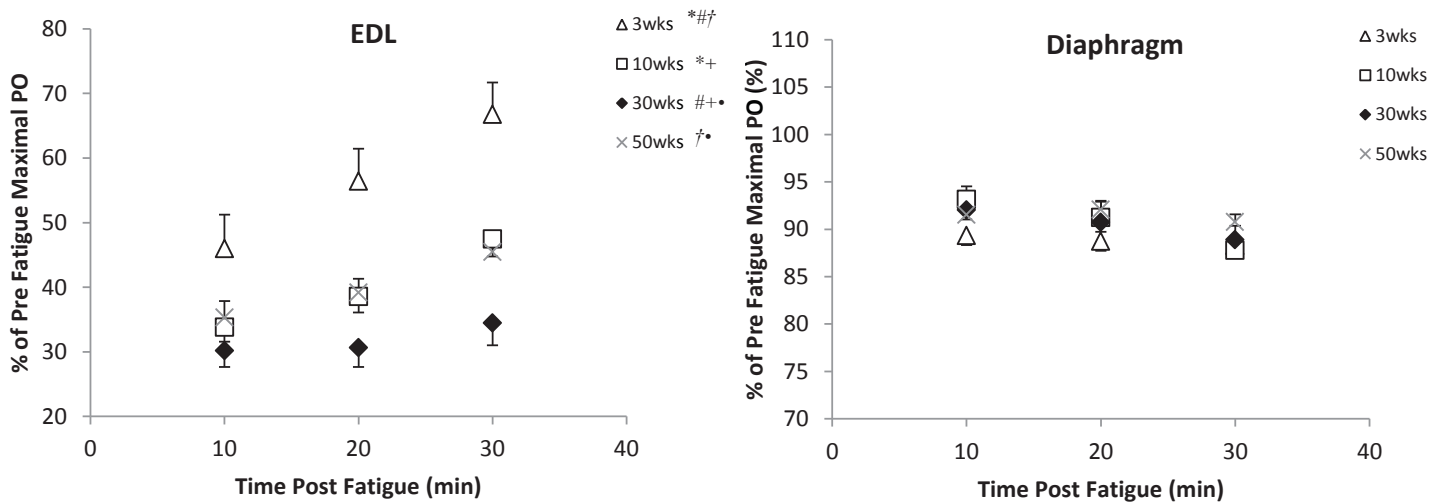


Figure 8 - The effect of age on mean recovery of power output of mouse EDL (A) and diaphragm (B) muscle following a protocol a repetitive work loop activity. There was an increase in muscle power output, over time, in EDL muscle with significantly greater recovery in 3 week EDL compared to all other age groups. Peak recovery of diaphragm muscle occurred after 10 minutes but there were no significant differences in the recovery pattern between age groups. [Data represented as mean  $\pm$  SE: n=10 for 10 & 30 weeks; n=9 for 50 weeks; n=8 for 3 weeks; wks = weeks of age; significant differences between age groups are indicated by them having common symbols]

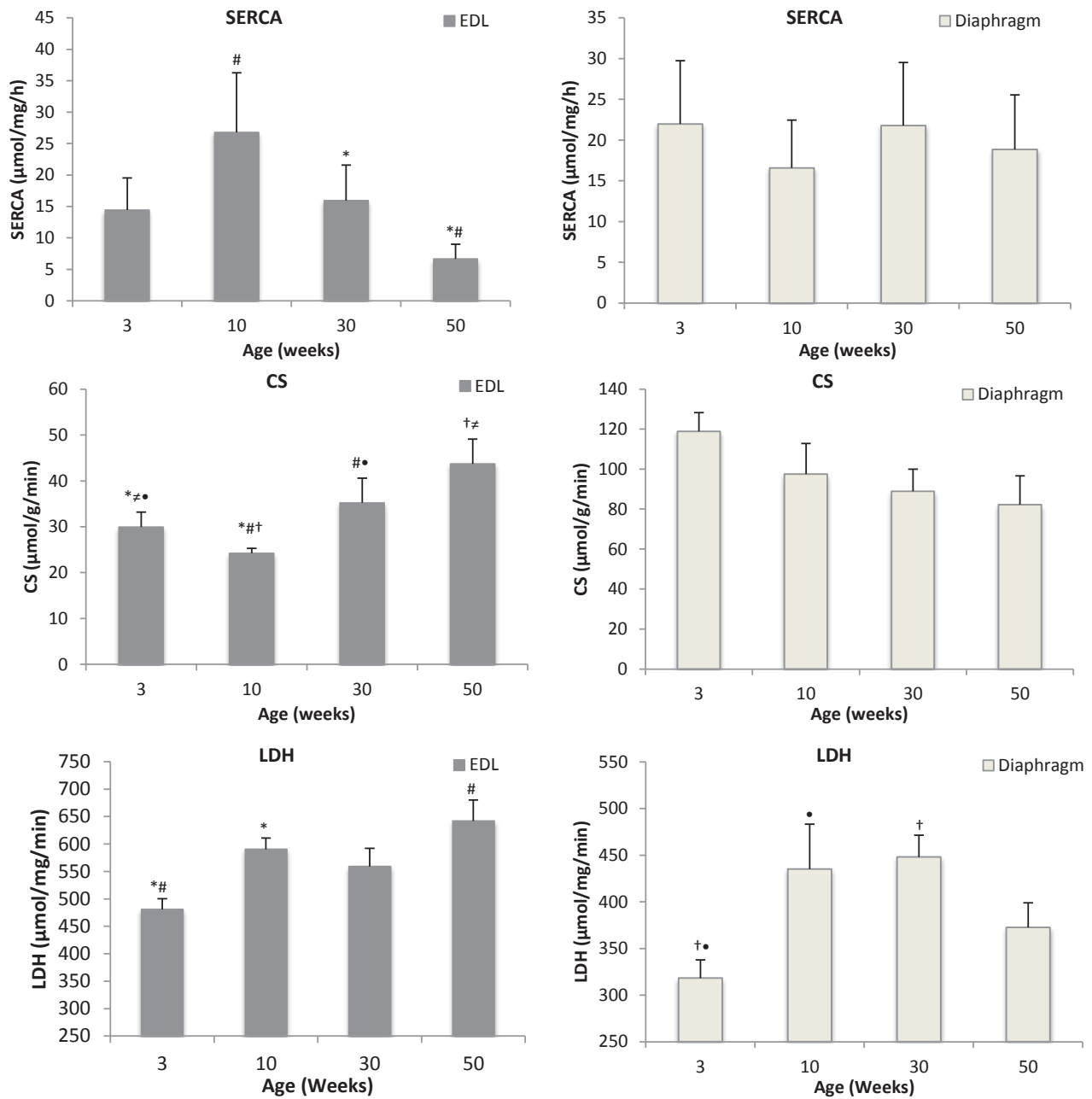


Figure 9 – The effect of age on EDL and diaphragm muscle activities of SERCA, CS and LDH

SERCA was significantly decreased and CS and LDH significantly increased in 50 week EDL muscle.

Diaphragm LDH activity was higher at 10 weeks when compared to that from 3 weeks old mice, but

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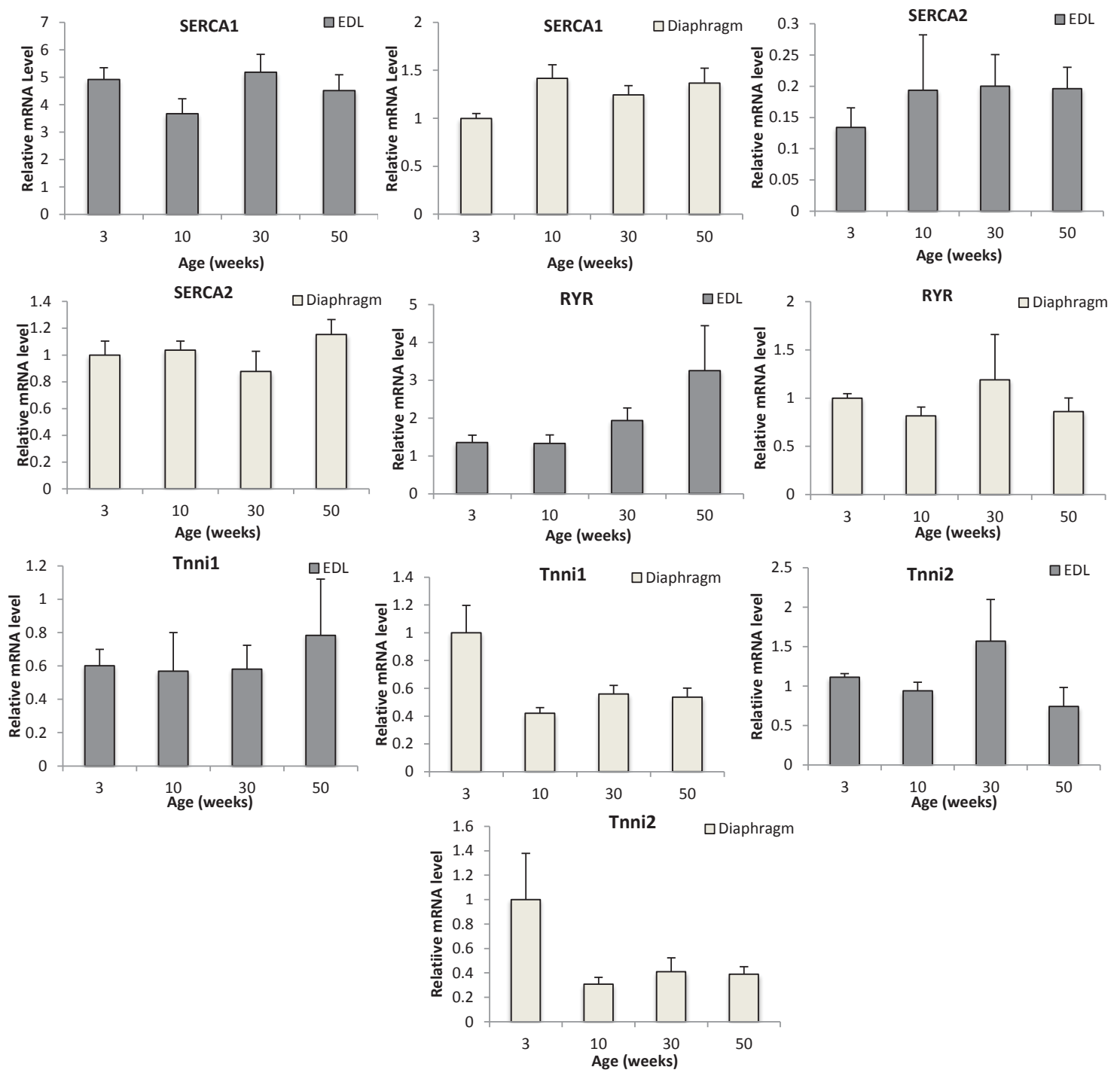


Figure 10 – There was no significant effect of increasing age on relative mRNA concentrations of SERCA1, SERCA2, RYR1, Tnni1, and Tnni2 in EDL and diaphragm muscle quantified by qRT-PCR [Data normalised to 3 week old mice and represented as mean  $\pm$  s.e.m: n=8-10 in each case]