The early effects of ageing on the mechanical performance of isolated locomotory (EDL) and respiratory (diaphragm) skeletal muscle using the work loop technique

Tallis, J. , James, R.S. , Little, A.G. , Cox, V. , Duncan, M.J. and Seebacher, F.

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

Jason Tallis[1] (✉), Rob S. James[1], Alexander G. Little[2], Val M. Cox[1], Michael J. Duncan[1], Frank Seebacher[2]

[1]Department of Biomolecular and Sport Sciences, James Starley Building, Coventry University, Priory Street, Coventry CV1 5FB, UK

[2]School of Biological Sciences, A08 University of Sydney, Science Road, Sydney, NSW, 2006, Australia

Effect of Ageing on Mechanical Properties of Skeletal Muscle

Email: tallisj2@uni.coventry.ac.uk

Abstract

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

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

Introduction

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

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

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

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

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

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

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

Materials and Methods

Animals

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

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

Dissection

Mice were killed by cervical dislocation in accordance with British Home Office Animals (Scientific Procedures) Act 1986, Schedule 1 and then weighed to determine body mass. EDL muscle was dissected from the right hind limb, and pinned out to approximately its resting length at room temperature (19-21°C). Throughout the dissection procedure the muscle was maintained in refrigerated and frequently changed oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution of composition (mM) NaCl 118; KCl 4.75; $MgSO_4$ 1.18; $NaHCO_3$ 24.8; KH_2PO_4 1.18; glucose 10; $CaCl_2$ 2.54; pH 7.55 at room temperature prior to oxygenation. Aluminium foil T-clips were wrapped around each tendon to minimise tendon slippage during muscle force production. Whole diaphragm muscle was dissected out, but only a ventral section of the costal diaphragm was used in the muscle mechanics protocol; aluminium foil T-clips were wrapped around the central tendon at one end, and at the opposing end two ribs anchoring the muscle were left intact.

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

Measurement of Mechanical Properties

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

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

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

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

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

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

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

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

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

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

Muscle Mass Measurements and Dimension Calculations

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

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

Biochemical analysis

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

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

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

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

Pre-validated TaqMan® Gene Expression Assays (Applied Biosystems, Australia) were used according to the manufacturer's instructions to quantify troponin 1 (tnni1; assay ID: Mm00502426_m1), troponin 2 (tnni2; Mm00437157_g1), Ca^{2+}-transporting-ATPase 1 (atp2a1; Mm01275320_m1), Ca^{2+}-transporting-ATPase 2 (atp2a2; Mm01201431_m1), ryanodine receptor 1 (ryr1; Mm01175211_m1) and elongation factor 1α2 (elf1a2; Mm00514649_m1) expression, with Elf1a2 as the housekeeping gene. We used Taqman Gene Expression Mastermix (Applied Biosystems, Australia) with the standard PCR protocol as recommended by the manufacturer. The cycle consisted of 95°C for 7 min, 40 cycles of 95°C for 20 s, 60°C for 1 min.

Statistical Analysis of Data

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

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

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

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

Results

Body & Muscle Mass

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

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

Maximal Isometric Twitch Stress

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

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

Maximal Isometric Tetanus Stress

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

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

Isometric Activation and Relaxation Times

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

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

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

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

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

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

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

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

Sustained Power Output

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

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

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

Recovery from Sustained Work loop Activation

There was a significant effect of age on the recovery of muscle power output post repetitive work loop activation in EDL muscle (Fig 8A; PERMANOVA d.f. = 3, 32; $F = 10.2$; $p < 0.001$). Mean recovery of EDL at 3 weeks of age was significantly greater than at 10, 30 and 50 weeks of age (Fig 8A; pair-wise $t > 2.51$; $p < 0.007$ in all cases). Recovery at 30 weeks of age was significantly reduced compared to 10 and 50 weeks of age (Fig 8A; pair-wise $t > 3.24$; $p < 0.006$).

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

Biochemical Analysis

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

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

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

Discussion

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

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

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

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

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

The current work extends the finding by Chan and Head [16], which concluded that a significant reduction in the tetanic stress of 20-22 month old female mice occurred without prevalent atrophy, by uniquely demonstrating an age related decline in dynamic muscle power that occurs in the absence of muscle atrophy and at a much younger age. Conversely, the muscle mass of EDL in the present study was significantly increased at 50 weeks and probably relates to a greater morphological size of the animal. Although evidence suggests that muscle mass is lost with ageing, the extent of such loss is variable and muscle group specific [14, 15, 55]. Brown and Hasser [15] suggested that this controversy may arise due to differences in the strain of rodents examined, the use of non-pathogen free animals and the age of the animals deemed to be aged. It has been suggested that significant muscle atrophy 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 Ca^{2+} handling proteins. The most documented of which is the uncoupling of
515 DHPR-ryanodine receptors resulting in a reduced 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].

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

Effect of Age on Sustained Muscle Power Output

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

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

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

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

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

Effect of Age on Recovery from Repetitive Stimulation

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

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

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

Limitations & Practical Implications of the Study

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

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

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

Having an improved understanding of the ageing response is important in the potential development of innovations to improve human health and quality of life [20]. The present study highlights significant reductions in skeletal muscle performance that occur at a relatively young age, and such effects are likely to be magnified in older age groups. Early ageing was associated with a greater loss of diaphragm force and power compared to 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*

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

Acknowledgements

The authors would like to thank Mark Bodycote and Bethan Grist for technical assistance during this project.

References

1. Agbulut O, Noirez P, Beaumont F, Butler-Browne G. Myosin heavy chain isoforms in postnatal muscle development of mice. *J Biology Cell* 95: 399-406, 2003.
2. Akhmedov D, Berdeux R. The effects of obesity on skeltal muscel regeneration. Front Physiol 17 1-12, 2013
3. Allen, DG, Lamb GD, Westerblad H. Skeletal muscle fatigue: Cellular mechanisms. *Physiol Rev* 88: 287-332, 2008.
4. Allman BL, Rice CL. Incomplete recovery of voluntary isometric force after fatigue is not affected by old age. *J Muscle and Nerve* 24: 1156-1167, 2001.
5. Alnaqeeb MA, Goldspink G. Changes in fibre type, number and diameter in developing and ageing skeletal muscle. *J Anat* 153: 31-45, 1986.
6. Altringham JD, Young IS. Power output and the frequency of oscillatory work in mammalian diaphragm muscle: the effects of animal size. *J Exp Biol* 157: 381-389, 1991.
7. Aniansson A, Hedberg M, Henning GB, Grimby G. Muscle morphology, enzymatic activity and muscle strength in elderly men: a follow up study. *J Muscle and Nerve* 9: 585-591, 1986.
8. Askew GN, Marsh R. The effects of length trajectory on the mechanical power output of mouse skeletal muscles. *J Exp Biol* 200 3119-3131, 1997
9. Askew GN, Young IS, Altringham JD. 'Fatigue of mouse soleus muscle, using the work loop technique. *J Exper Biol* 200: 2907-2912, 1997.
10. Backman E, Johansson V, Hager B, Sjoblom P, Henriksson KG. Isometric muscle strength and muscular endurance in normal persons aged between 17 and 70 years. *Scand J Rehabil Med* 27 (2), 109-117, 1995.
11. Bemben MG, Massey BH, Bemben DA, Misner JE, Boileau RA. 'Isometric intermittent endurance of four muscle groups in men aged 20-74 yr.' *J Med Sci Sport Exerc* 28: 145-154, 1996.
12. Berchtold MW, Heinrich B, Munterner M. Calcium ion in skeletal muscle: Its crucial role for muscle function, plasticity, and disease. *Physiol Rev* 80: 1215-1265, 2000.
13. Bilodeau M, Henderson TK, Nolta, BE, Pursley PJ, Sandford GL. 2001 'Effect of ageing on fatigue characteristics of elbow flexor muscles during sustained sub maximal contraction. *J Appl Physiol* 91: 2654-2664, 2001.
14. Brooks SV, Faulkner JA. Contractile properties of skeletal muscle from young, adult and aged mice. *J Physiol* 404: 71-8, 1988.
15. Brown M, Hasser EM. Complexity of age-related changes in skeletal muscle. *J Gerontol A Biol Sci Med Sci* 51: 117-123, 1996.
16. Chan S, Head SI. Age- and gender-related changes in contractile properties of non-atrophied EDL muscle. *PLoS ONE* 5, 2010.
17. Coggan AR, Spina RJ, King DS, Rogers MA, Brown M, Nemeth PM, Holloszy JO. Histochemical and enzymatic comparison of the gastrocnemius muscle of young and elderly men and women. *J Gerontol* 47: 71-76, 1991.
18. Davies CT, Thomas DO, White MJ. Mechanical properties of young and elderly human muscle *Acta Med Scand Suppl* 711: 219-226, 1986.

19. Delbono O, O'Rourke KS, Ettinger WH. Excitation-calcium release uncoupling in aged single human skeletal muscle fibres *J Membr Biol* 148: 211-222, 1995.

20. Deschenes M. Effects of aging on muscle fibre type and size. *J Sports Med* 34: 809-824, 2004.

21. Doherty TJ. Invited review: aging and sarcopenia. *J Appl Physiol* 95: 1717-1727, 2003.

22. Drummond GB, Vowler SL. Different tests for a difference: how do we do research? *J Physiol* 590: 235-238, 2011.

23. Gonzalez E, Delbono O. Recovery from fatigue in fast and slow intact skeletal muscle fibres from aging mice. *J Muscle & Nerve* 24: 1219-1224, 2001a

24. Gonzalez E, Delbono O. Age-dependent fatigue in single intact fast- and slow fibres from mouse EDL and soleus skeletal muscle. *Mech Ageing Dev* 122: 1019-1032, 2001b.

25. Gonzalez E, Messi ML, Delbono O. The specific force of single intact extensor digitorum longus and soleus mouse muscle fibres declines with aging. *J Membrane Biol* 178: 175-183, 2000.

26. Gosselin LE, Johnson BD, Sieck GC. Age-related changes in diaphragm contractile properties and myosin heavy chain isoform. *Am J Respir Crit Care Med* 150: 178-178, 1994.

27. He J, Watkins S, Kelley D.E. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fibre type in type 2 diabetes. *Diabetes* 50 817-823, 2001.

28. Higgins MF, Tallis J, Price MJ, James RS. The effects of elevated levels of sodium bicarbonate ($NaHCO_3$) on the acute power output and time to fatigue of maximally stimulated mouse soleus and EDL muscles. *Eur J Appl Physiol* 113 1331-1341, 2013.

29. Hunter SK, Thompson MW, Ruell PA, Harmer AR, Thom JM, Gwinn TH, Adams RD. 'Human skeletal sarcoplasmic reticulum Ca^{2+} uptake and muscle function with aging and strength training. *J Appl Physiol* 86: 1858-1865, 1999.

30. Izquierdo M, Hakkinen K, Anton A, Garrues M, Ibanez J, Ruesta M, Gorostiaga EM. Maximal strength and power, endurance performance, and serum hormones in middle-aged and elderly men. *J Med Sci Sport Exe* 33: 1577-1578, 2001.

31. James RS, Altringham JD, Goldspink DF. The mechanical properties of fast and slow skeletal muscles of the mouse in relation to their locomotory function. *J Exp Biol* 198: 491-502, 1995.

32. James RS, Kohlsdorf T, Cox VM, Navas CA. 70μM caffeine treatments enhances *in vitro* force and power output during cyclic activities in mouse extensor digitorum longus muscle. *Euro J Appl Physiol* 95: 74-82, 2005.

33. James RS, Walter I, Seebacher F. Variation in expression of calcium-handling proteins is associated with inter-individual differences in mechanical performance of rat (*Rattus norvegicus*) skeletal muscle. *J Exp Biol* 214: 3542-3548, 2011.

34. James RS, Young IS, Cox VM, Goldspink DF, Altringham JD. Isometric and isotonic muscle properties as determinants of work loop power output. *Euro J Physiol* 432: 767-774, 1996.

35. Josephson RK. Mechanical power output from striated muscle during cyclical contraction. *J Exp Biol* 114: 493-512, 1985.

36. Kent-Braun JA, Ng AV, Doyle JW, Towse TF. Human skeletal muscle response vary with age and gender during fatigue due to incremental isometric exercise. *J Appl Physiol* 93: 1813-1823, 2002.

37. Klitgaard H, Mantoni M, Schiaffino S, Ausoni S, Gorza L, Laurent-Winter C, Schnohr P, Saltin B. Function, morphology and protein expression of ageing skeletal muscle: a cross-sectional study of elderly men with different training backgrounds. *J Acta Physiol Scand* 140: 41-54, 1990.

38. Krivickas LS, Suh D, Wilkins J, Hughes VA, Roubenoff R, Frontera WR. 'Age- and gender-related differences in maximum shortening velocity of skeletal muscle fibres. *Am J Physical Med Rehabil* 80: 447-455, 2001.

39. Lang PL, White WJ. Growth**,** development**,** life span and select lesion incidence in the aging CD-1 mouse. *Product Literature,* Charles River Laboratories.

40. Larsson L, Salviati G. Effects of age on calcium transport activity of sarcoplasmic reticulum in fast- and slow-twitch rat muscle fibres. *J Physiol* 419: 253-264, 1989.

41. Lennmarken C. Bergman T, Larsson J, Larsson LE. Skeletal muscle function in man: force, relaxation rate, endurance and contraction time-dependence on sex and age. *J Clin Physiol* 5: 243-255, 1985.

42. Lexell J. Human ageing, muscle mass, and fibre type composition. *J Gerontol A: Bio Sci Med Sci* 50: 11-6, 1995.

43. Lindstrom B, Lexell J, Gerdle B, Downham D. Skeletal muscle fatigue and endurance in young and old men and women. *J Gerontol A Bio Sci Med Sci*52: 59-66, 1997.

44. Luff AR, Atwood HL Changes in the sarcoplasmic reticulum and transverse tubular system of fast and slow skeletal muscles of the mouse during postnatal development. *J Cell Biol* 51: 369-383, 1971.

45. Lynch GS, Hinkle RT, Chamberlain JS, Brooks SV, Faulkner. Force and power output of fast and slow skeletal muscles from mdx mice 6-28 months old. *J Physiol* 535 591-600, 2001

46. Martini, F., William, C., and Garrison, C.W. (2000) *Fundamentals of anatomy & physiology.* Benjamin-Cumming Publishing Company, San Francisco.

47. Méndez J, Keys A. Density and composition of mammalian muscle. *Metab* 9,184-188, 1960.

48. Metter EJ, Conwit R, Tobin J, Fozard JL. Age-associated loss of power and strength in the upper extremities in women and men. *J Gerontol A Bio Sci Med Sci* 52A: 267-276, 1997.

49. Moran AL, Warren GL, Lowe DA. Soleus and EDL muscle contractility across the lifespan of female C57Bl/6 mice. *J Exp Gerontol* 40: 966-975, 2005

50. Murray MP, Gardner GM, Mollinger LA, Sepoc SB. Strength of isometric and isokinetic contractions: Knee muscles of men aged 20-86. *J Physical Therapy* 60: 412-419, 1980.

51. Murray MP, Duthie EH, Gambert SR, Sepic SB, Mollinger LA. Age-related differences in knee muscle strength in normal women. *J Gerontol* 40: 275-280, 1985.

52. Narayanan N, Jones DL, Xu A, Yu JC. Effects of aging on sarcoplasmic reticulum function and contraction duration in skeletal muscle of the rat. *Am J Physiol* 271: 1032-1040, 1996.

53. Navarro A, Lopez-Cepero JM, Sanchez del Pino MJ. Skeletal Muscle and Ageing. *Frontiers in Bioscience* 6: 26-44, 2001.

54. Ottenheijm CAC, Heunks LMA, Dekhuijzen RPN. Diaphragm adaptations in patients with COPD. *J Respir Res* 9: 12, 2008.

55. Pagala MK, Ravindran K, Namba T, Grob D. Skeletal muscle fatigue and physical endurance of young and old mice. *J Muscle and Nerve* 21: 1729-1739, 1998.

56. Polkey MI, Kyroussis D, Hamnegard CH, Mills GH, Green M, Moxham J. 'Diaphragm strength in chronic obstructive pulmonary disease. *Am J Respir Crit Care Med* 154: 1310-1317, 1996.

57. Raj IS, Bird SR, Shield AJ. Aging and the force-velocity relationship of muscles. *J Exp Gerontol* 45: 81-90, 2010.

58. Renganathan M, Messi ML, Delbono O. Dihydropyride receptor-ryanodine receptor uncoupling in aged skeletal muscle. *J Membrane Biol* 157: 247-253, 1997

59. Seebacher F, Guderley H, Elsey RM, Trosclair PL. Seasonal acclimatisation of muscle metabolic enzymes in a reptile (Alligator mississippiensis). *J Exp Biol* 206: 1193–1200, 2003.

60. Schiaffino S, Margreth A. Coordination development of the sarcoplasmic reticulum and the t system during postnatal differentiation of rat skeletal muscle. *J Cell Biol* 41: 855-875, 1969.

61. Short KR, Bigelow, ML, Kahl J, Sing R, Coenen-Schimke J, Raghavakaimal S, Nair KS. Decline in skeletal muscle mitochondrial function with aging in humans. *Proc Natl Acad Sci U S A* 102: 5618-5623, 2005.

62. Skelton DA, Greig CA, Davies JM, Young A. Strength, power and related functional ability of health people aged 65-89 years. *J Age and Ageing* 23: 371-377, 1994.

63. Strohacker K, Breslin WL. Carpenter KC, McFarlin BK. Aged mice have increased inflammatory monocyte concentration and altered expression of cell-surface functional receptors. *J Biosci* 37 55-62, 2012.

64. Tallis J, James RS, Cox VM, Duncan MJ. The effect of physiological concentrations of caffeine on the power output of maximally and sub maximally stimulated mouse EDL (fast) and soleus (slow) muscle. *J Appl Physiol* 112: 64-71, 2012.

65. Tallis J, James RS, Cox VM, Duncan MJ. The effect of a physiological concentration of caffeine on the endurance of maximally and submaximally stimulated mouse soleus muscle. *J Physiol Sci* 63: 125-132 2013.

66. Thompson LV, Brown M. Age-related changes in contractile properties of single skeletal fibres from the soleus muscle. *J Appl Physiol* 86: 881-886, 1999.

67. Unger RH. Minireview: Weapons of lean body mass destruction: The role of ectopic lipids in the metabolic syndrome. Endocrinology 144 5159-5165, 2003

68. Viner RI, Ferrington DA, Willaims TA, Bigelow DJ, Schoneich C. Protein modification during biological aging: slective tyrosine nitration of the SERCA2a isoform of the sarcoplamsic reticulum Ca2+-ATPase in skeletal muscle*. Biochem J* 340 657-669, 1999.

69. Walter I, Seebacher F. Molecular mechanisms underlying the development of endothermy in birds (Gallus gallus): a new role of PGC-1 alpha? *AM J Physiol Intergr Comp Physiol* 293: 2315-2322, 2007.

70. Warrington JS, Poku JW, Von Moltke LL Shader, RI, Harmatz, JS, Greenblatt DJ. Effects of Age on In Vitro Midazolam Biotransformation in Male CD-1 Mouse Liver Microsomes. *J Pharmacol Exp Ther* 292 1024-1031, 2000.

71. Warrington JS, Von Moltke LL, Harmatz JS, Shader RI, Greenblatt DJ. The Effect Of Age On Sildenafil Biotransformation In Rat And Mouse Liver Microsomes. *Drug Metab Dispos* 31 1306-1309, 2003.

72. Williams GN, Higgins MJ, Lewek MD. Ageing skeletal muscle physiologic changes and the effects of training. *J Physical Therapy* 82: 62-68, 2002.

73. Zhang YL, Kelsen SG. Effects of aging diaphragm contractile function in golden hamsters. *Am Rev Respir Dis*146: 1396-1401, 1990.

74. Zierath JR, Hawley JA. Skeletal muscle fibre type: influence on contractile and metabolic properties. *PLoS Biology* 2: 337-384, 2004.

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

		Age (Weeks)			
Muscle		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

Data represented as mean±s.e.m: n = 10 for each group. Data for EDL represents whole muscle mass and for diaphragm the mass represents the section of the muscle used in the evaluation of mechanical performance.

Figure Captions

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 ± 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 ± s.e.m: n=10 in each case; significant differences between age groups are indicated by them having common symbols]

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

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

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 ± s.e.m: n=10 in each case; wks = weeks of age; significant differences between age groups are indicated by them having common symbols]

Figure 6. - The effect of age on typical work loop shapes of mouse EDL muscle during repetitive activation at 10Hz 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 (0.2s of the protocol), 10 (1s) and 18 (1.8s) 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 to 50 week old EDL. EDL muscles from this oldest age group were associated with the poorest fatigue resistance.

Figure 7. - The effect of age on typical work loop shapes of mouse diaphragm muscle during repetitive activation at 7Hz 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 ± 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 beyond this there were limited changes in the measured enzyme activities. [Data represented as mean ± s.e.m: n=8-10 in each case; significant differences between age groups are indicated by them having common symbols]

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 ± s.e.m: n=8-10 in each case]

Figures

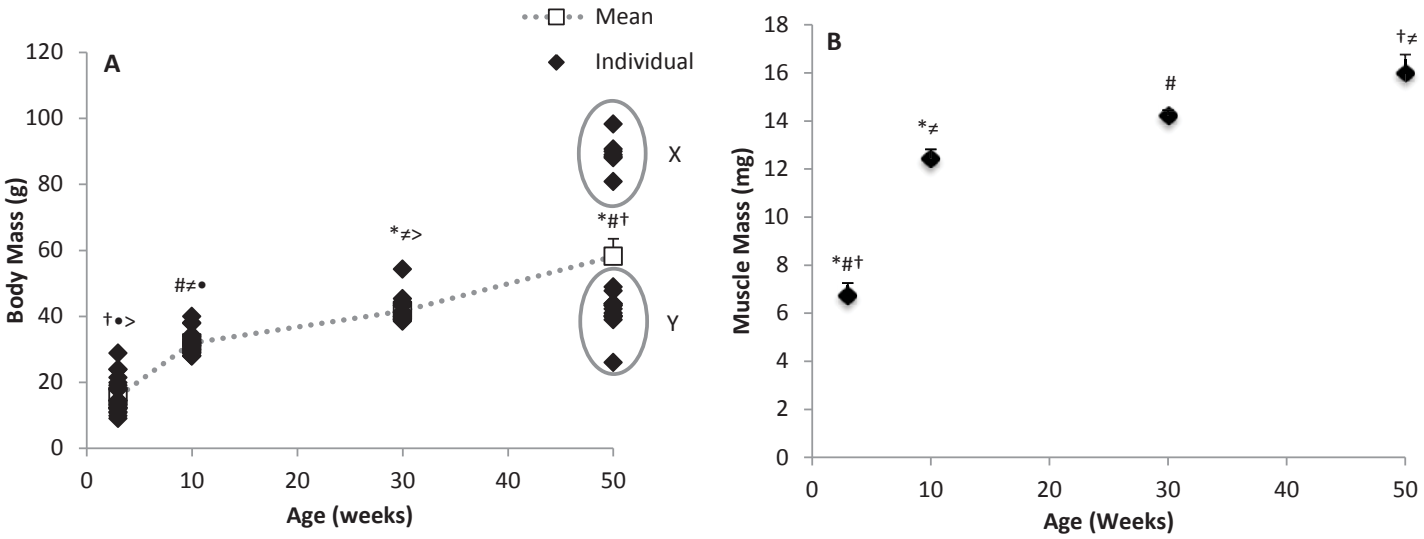

Figure 1. – Increasing age resulted in greater mean body mass of CD1 mice (**A**) and higher EDL muscle mass (**B**). A subdivision in the 50 week data is highlighted by **X**&**Y**. Data labelled **X** represent 50 week old mice with body mass greater than 70g, whereas data labelled **Y** represents 50 week old mice with body mass below 50g [Data represented as mean ± 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]

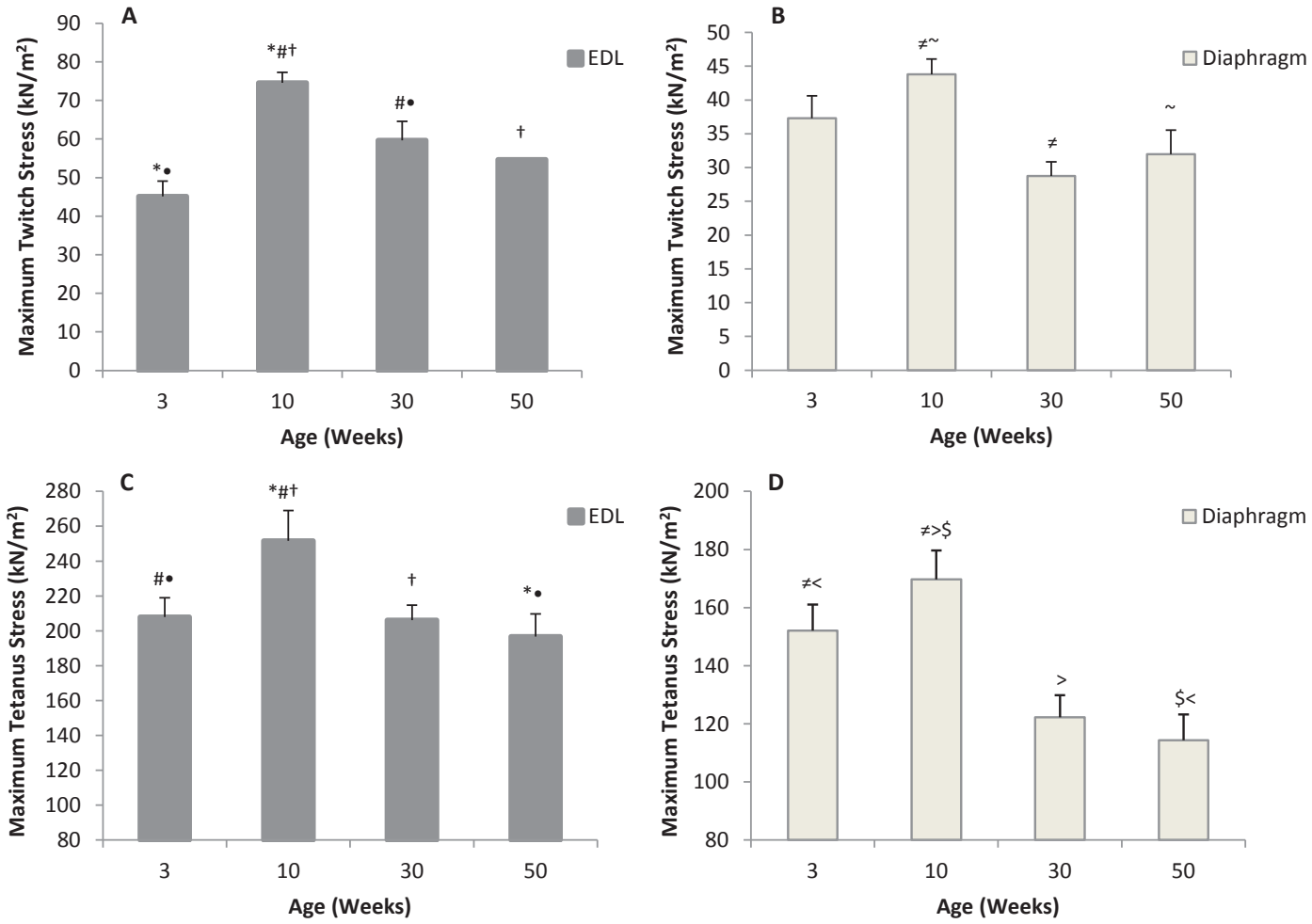

Figure 2. – The effect of age on mean maximal isometric twitch and tetanus stress in mouse EDL (A & C) and diaphragm (B & D) muscle. Increasing age, from maturity, generally caused a decrease in maximal isometric twitch and tetanus stress in EDL and diaphragm muscle. Maximal twitch and tetanus stress 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 ± s.e.m: n=10 in each case; significant differences between age groups are indicated by them having common symbols]

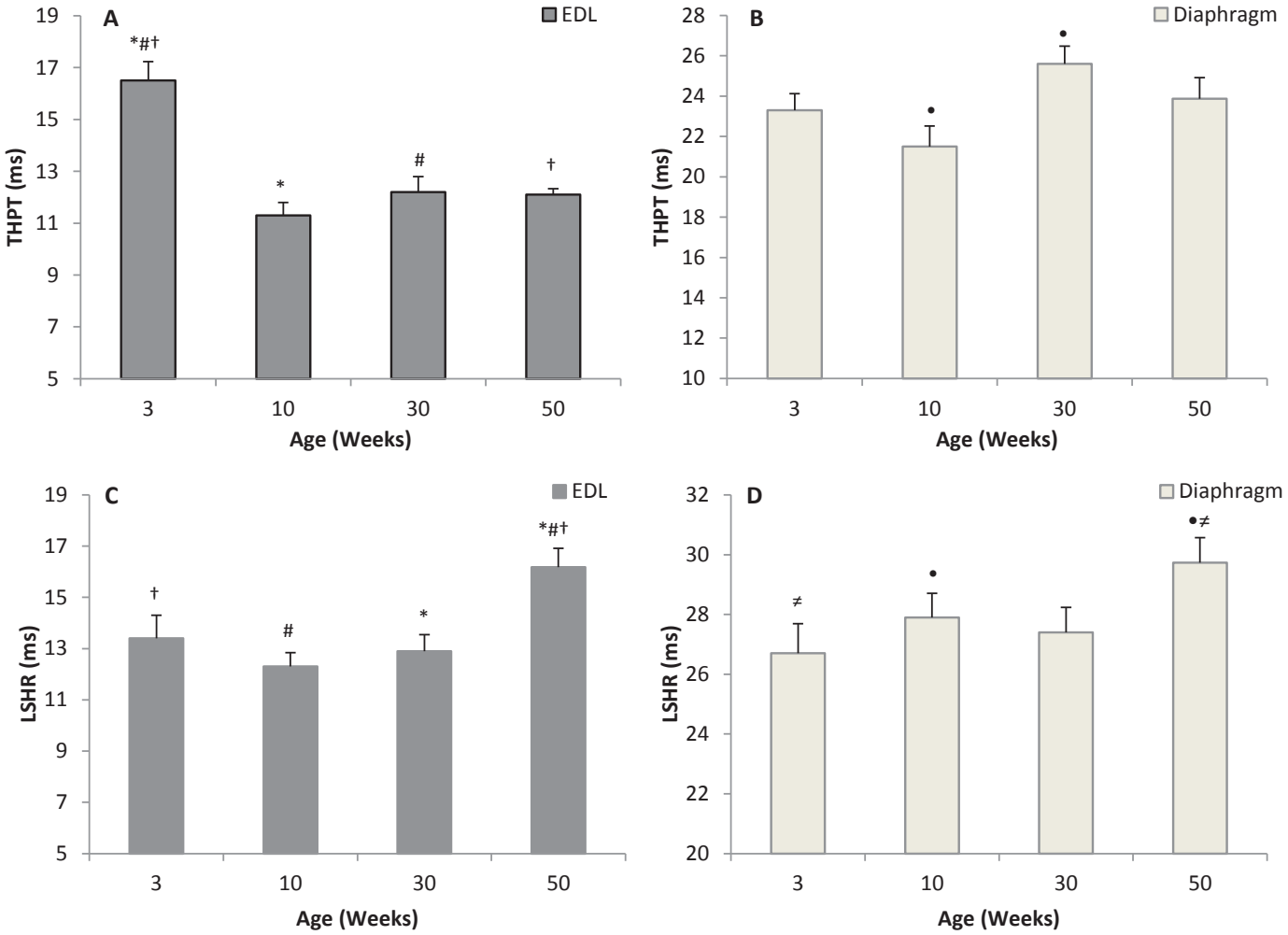

Figure 3 – The effect of age on mean isometric tetanus muscle activation time (THPT; time to half peak tetanus) and relaxation time (LSHR; last stimulus to half tetanus relaxation) in mouse EDL (A & C) and diaphragm muscle (B & D). THPT was significantly longer in 3 week old EDL, but beyond this there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

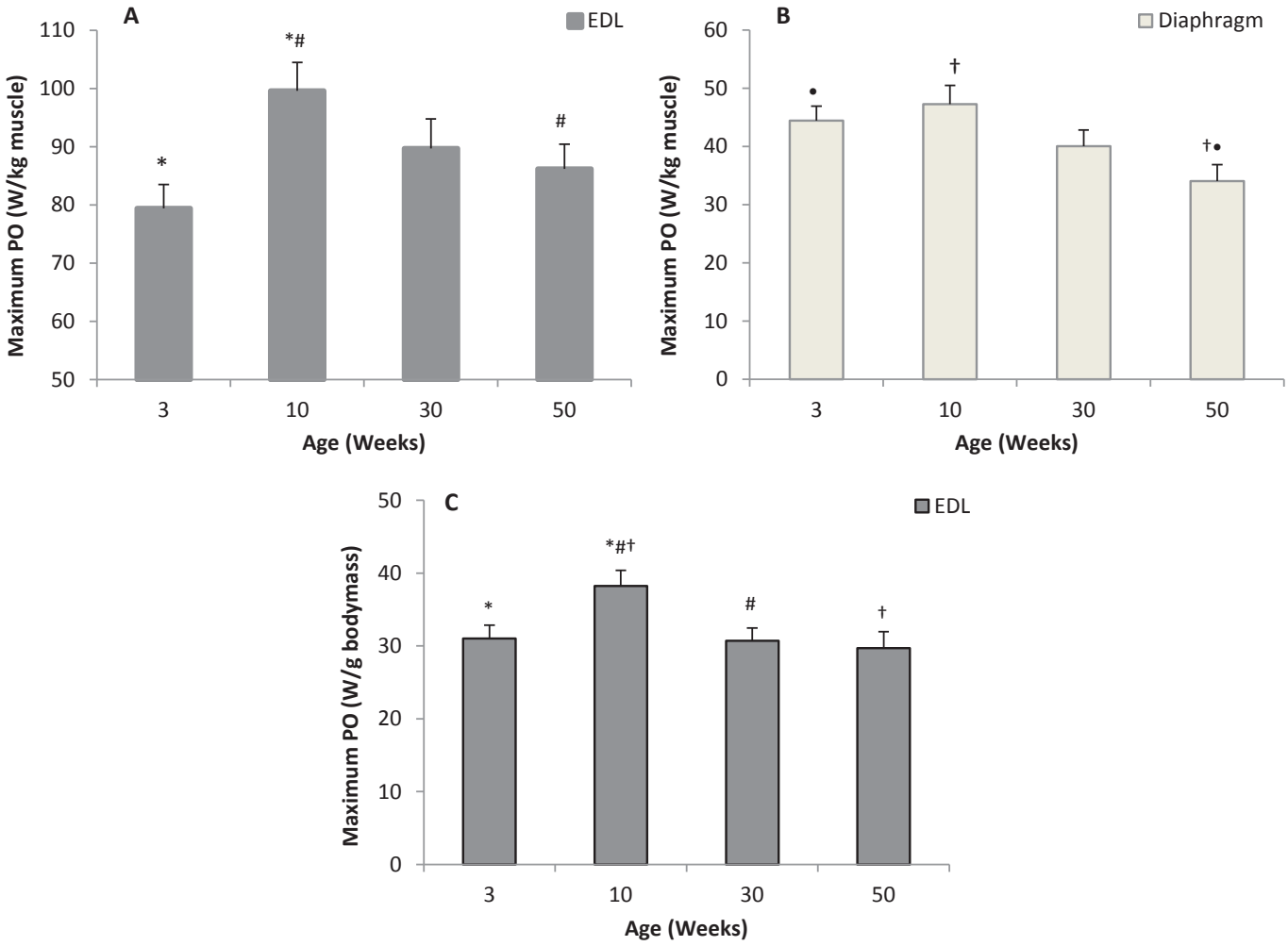

Figure 4. - The effect of age on mean maximal work loop power output plotted as Watts per kilogram muscle mass for mouse EDL (A) and diaphragm (B) muscles, and Watts per gram body mass for EDL (C). Maximal power output was achieved at 10 weeks of age in both EDL and diaphragm muscle and beyond this,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

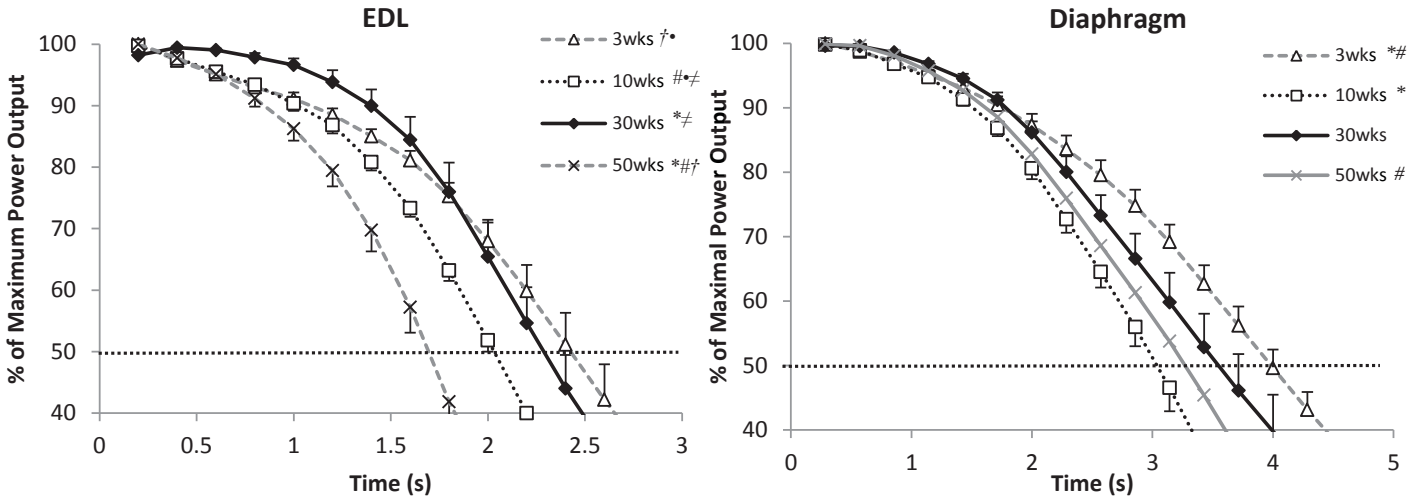

Figure 5. - The effect of age on sustained muscle power output during repetitive work loop activation in mouse EDL and diaphragm muscle. The ability to maintain power through repetitive activation was muscle specific, however there was a general pattern of age related changes with greatest maintenance 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 ± s.e.m: n=10 in each case; wks = weeks of age; significant differences between age groups are indicated by them having common symbols]

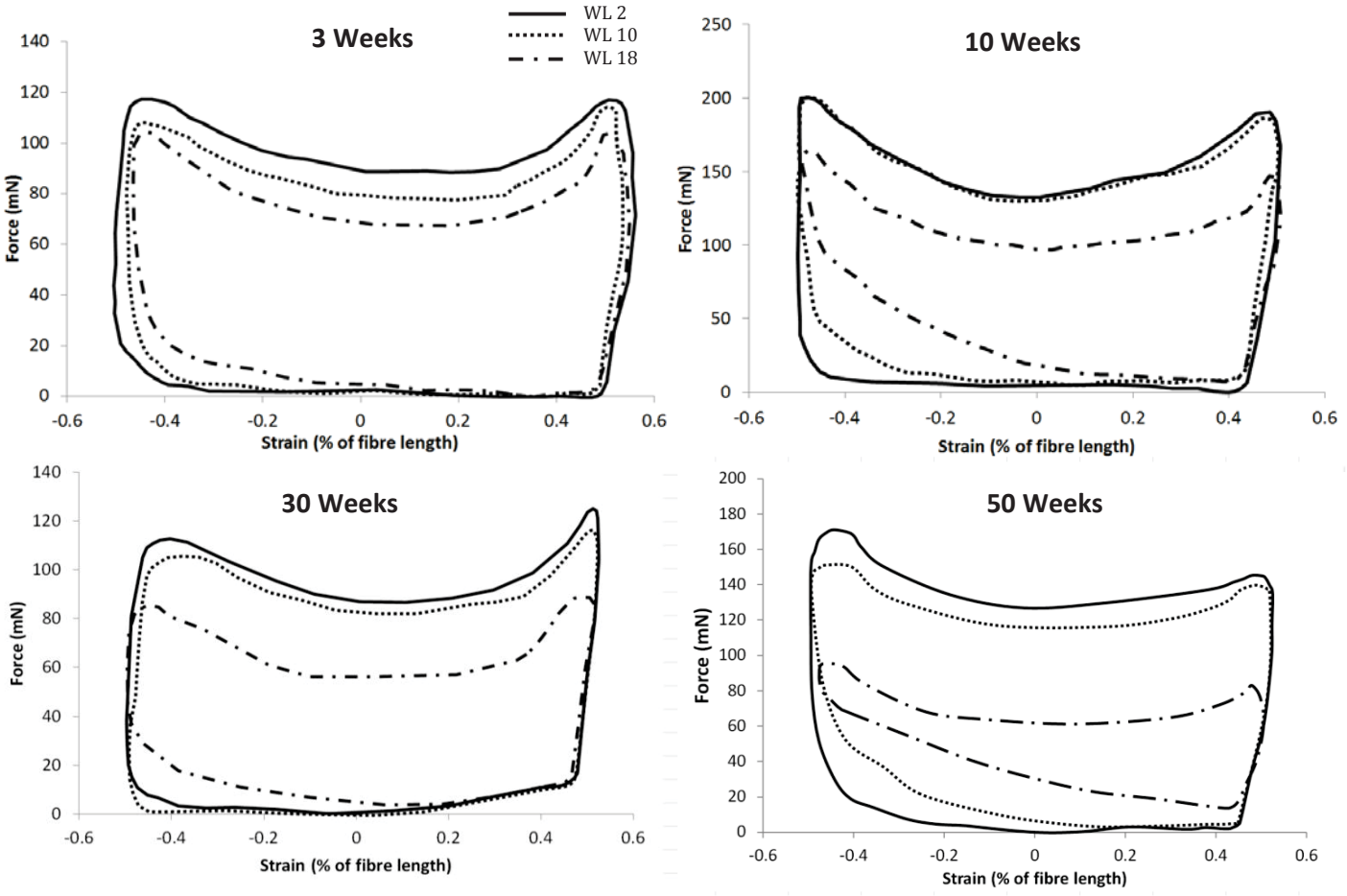

Figure 6. - The effect of age on typical work loop shapes of mouse EDL muscle during repetitive activation at 10Hz cycle frequency for 3 week old mice, 10 week old mice, 30 week old mice and 50 week old mice. The figures depict work loops 2 (0.2s of the protocol), 10 (1s) and 18 (1.8s) of the fatigue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

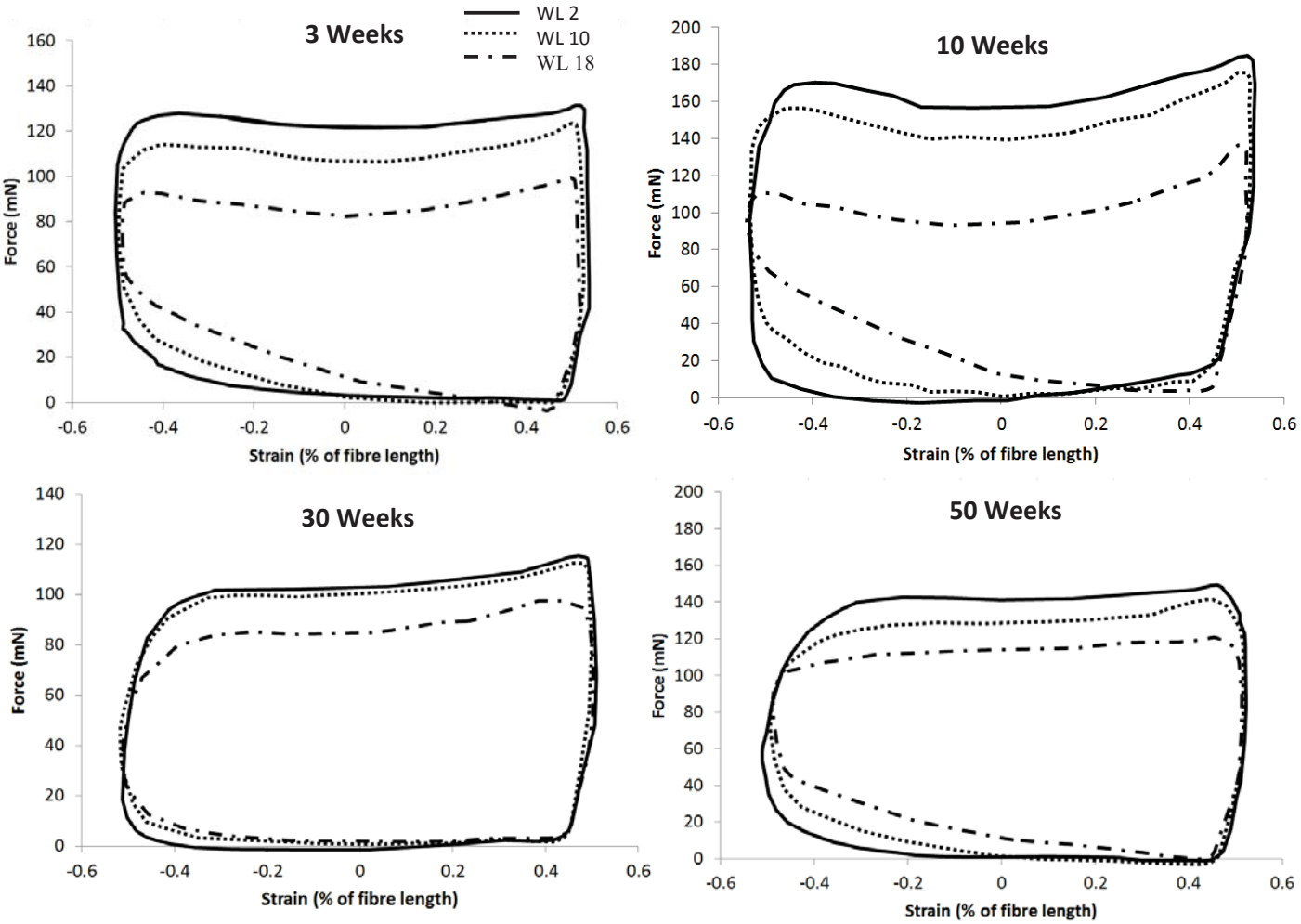

Figure 7. - The effect of age on typical work loop shapes of mouse diaphragm muscle during repetitive activation at 7H cycle frequency for 3 week old mice, 10 week old mice, 30 week old mice and 50 week old mice. The figures depict work loops 2 (0.29s of the protocol), 10 (1.43s) and 18 (2.57s) of the 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.

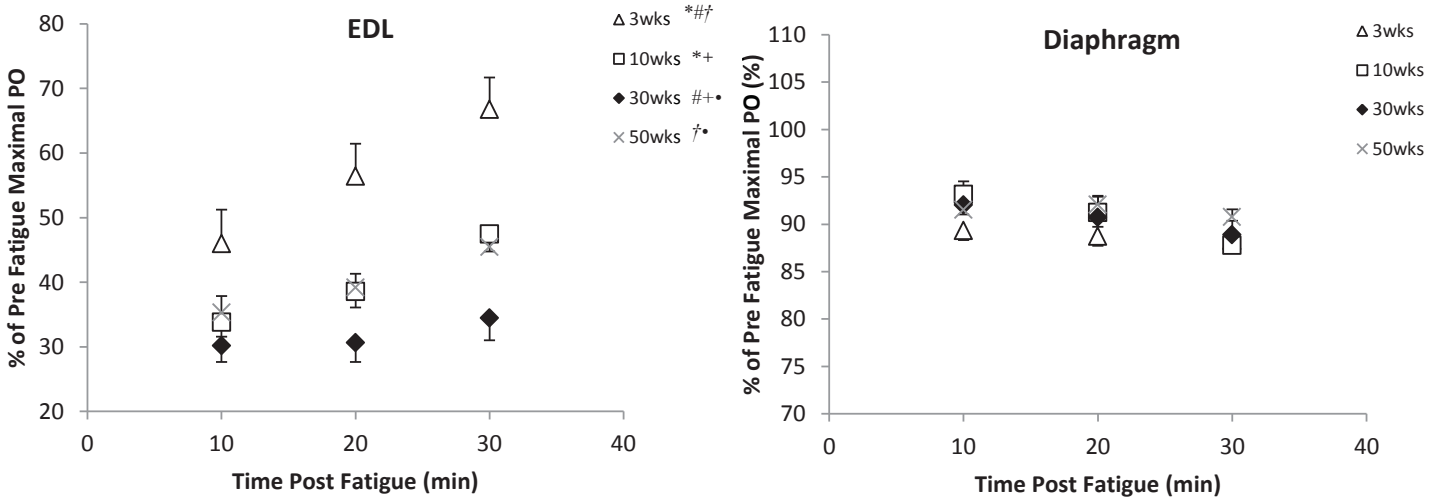

Figure 8 - The effect of age on mean recovery of power output of mouse EDL (A) and diaphragm (B) muscle following a protocol a repetitive work loop activity. There was an increase in muscle power output, over time, in EDL muscle with significantly greater recovery in 3 week EDL compared to all other 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 ± 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]

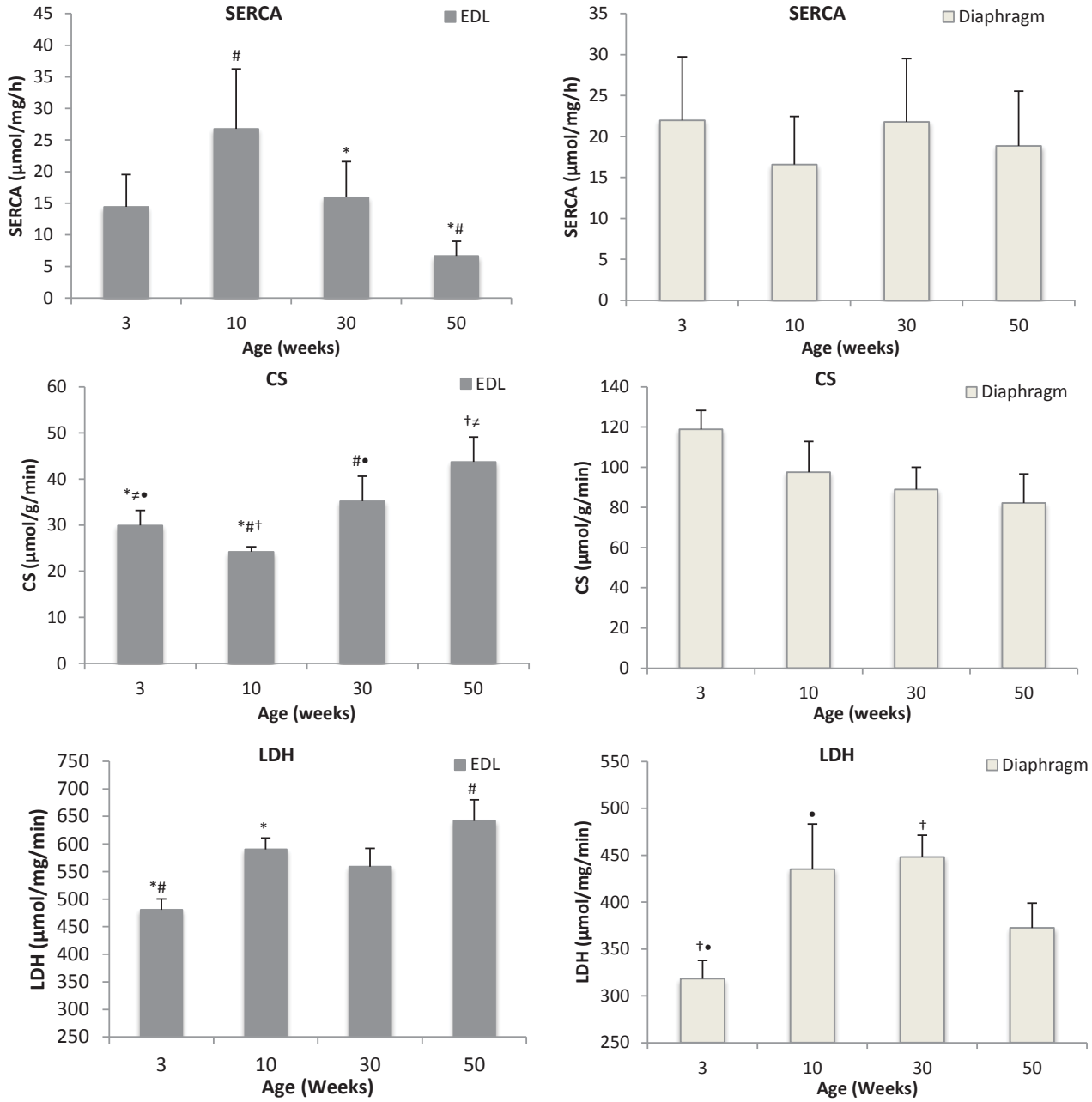

Figure 9 – The effect of age on EDL and diaphragm muscle activities of SERCA, CS and LDH

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 beyond this there were limited changes in the measured enzyme activities. [Data represented as mean ± s.e.m: n=8-10 in each case; significant differences between age groups are indicated by them having common symbols]

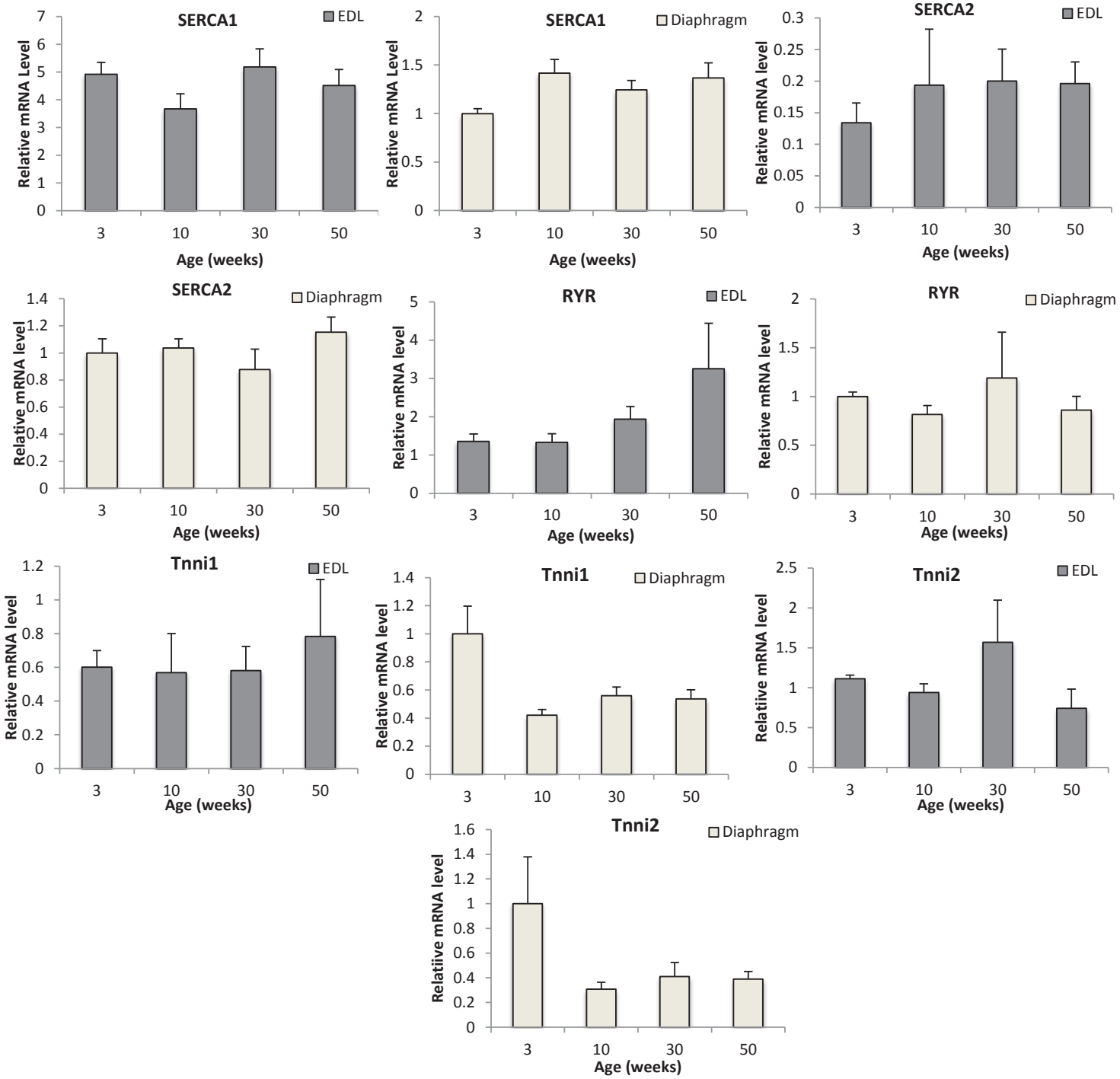

Figure 10 – There was no significant effect of increasing age on relative mRNA concentrations of SERCA1, SERCA2, RYR1, Tnni1, and Tnni2 in EDL and diaphragm muscle quantified by qRT-PCR [Data normalised to 3 week old mice and represented as mean ± s.e.m: n=8-10 in each case]