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

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Tallis J, James RS, Little AG, Cox VM, Duncan MJ, Seebacher F. Early effects of ageing on the mechanical performance of isolated locomotory (EDL) and respiratory (diaphragm) skeletal muscle using the work loop technique. Am J Physiol Regul Integr Comp Physiol 307: R670–R684, 2014. First published July 2, 2014; doi:10.1152/ajpregu.00115.2014.—Previous isolated muscle studies examining the effects of ageing on contractility have used isometric protocols, which have been shown to have poor relevance to dynamic muscle performance in vivo. The present study uniquely uses the work-loop technique for a more realistic estimation of in vivo muscle function to examine changes in mammalian skeletal muscle mechanical properties with age. Measurements of maximal isometric stress, activation and relaxation time, maximal power output, and sustained power output during repetitive activation and recovery are compared in locomotory extensor digitorum longus (EDL) and core diaphragm muscle isolated from 3-, 10-, 30-, and 50-wk-old female mice 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 than EDL muscle 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 rationalize previously reported equivocal results from examination of 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 suggests that although there is some evidence of a more oxidative fiber type, this is not the primary contributor to the early age-related reduction in muscle contractility.

The age-related reduction in skeletal muscle function has been studied at length and is primarily associated with a loss of muscle mass and strength and a slowing of contractile function, which greatly reduce mobility and, subsequently, the quality of life in elderly populations (72). However, muscle atrophy and the associated decline in skeletal muscle performance can occur as early as 25 yr 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 (maximum force in a single attempt) and power (the rate at which work is done; force produced × 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 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 extensor digitorum longus (EDL) without changes in the force-velocity relationship; 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 sustain performance over time) have demonstrated equivocal findings in vivo human (10, 11, 30, 40, 43) and isolated mammalian (16, 24, 55, 73) muscle research. 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 of 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-mo-old hamsters stimulated via repeated isometric tetanic contraction. In contrast, González and Delbono (24) concluded that the reduction in mechanical performance was not related to changes in fatigability of EDL and soleus muscle from 20- to 24-mo-old mice. Further ambiguity is added by examination of the findings of Pagala et al. (55), who reported that, despite a decline in whole animal endurance performance in aged (34- to 36-mo-old) mice, the fatigue resistance of tetanic stress (force per cross-sectional area) was significantly increased. Their research assessed the ability of muscle to sustain performance via repeated
twitch behavior, and 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 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 findings of previous ageing research (14, 16, 23–25, 34, 35) are related to whole animal performance. There is a dearth of in vitro studies of the effect of ageing on muscle power, and estimations of power output from isometric and isovelocity data, as reported by 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, lengthen before subsequent contraction. As for in vivo power-producing muscles, the work-loop technique uses waveforms and stimulation parameters that more closely replicate those used in vivo to consider muscle force production over dynamic contractions, accounting for the interaction of force production during shortening, resistance to muscle relengthening, and changes in activation and relaxation time (31, 32, 34, 35).

Much of the ageing research on skeletal muscle activity in rodents compares a physiologically mature population with an aged population, and relatively little is known about the rate of decline between maturity and old age. The present study implements the work-loop method to determine the time course of age-related changes in mechanical properties of mouse EDL muscles [typically type IIX (9.3%), IIB (86.8%), and I (3.9%) fibers at 90 days (1)] and diaphragm [typically type IIa (43.6%), IIX (34.6%), IIB (6.2%), and I (15.6%) fibers at 90 days (1)]. It is hypothesized 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, and 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 because of its underlying functional significance. The reduction in fast muscle fiber types is commonplace in ageing skeletal muscle (5, 7, 17); thus it is considered that EDL muscle will age more quickly. 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 muscle.

MATERIALS AND METHODS

Animals

The Ethics Committee of Coventry University approved the use of animals in this study. Female CD-1 mice (Charles River, UK) kept in conventional, not specific pathogen-free, conditions were bred and housed 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] and water ad libitum. From birth, mice were housed in groups of eight without access to running wheels and sampled at 3, 10, 30, and 50 wk 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 wk, when they are believed to be adults. Hence, muscle dissected from 3-wk-old mice was used to represent growth. Lang and White (39) demonstrated a >85% survival rate for CD-1 mice at 50 wk of age; however, beyond this point, mortality rate increased more rapidly and, at 18 mo, was ∼50%. Previous research examining the ageing effect on skeletal muscle mechanical performance has commonly used 18- to 24-mo-old mice from different strains (C57BL/6, DBA, and FVB) to represent old age (14, 23–25). Similarly, 18- to 24-mo-old CD-1 mice have been used as animal models for ageing research (63, 70, 71). Gonzalez et al. (25), who investigated the reduction in specific force of EDL and soleus muscle fibers, used 12-mo-old mice to represent a “middle-aged” group. In light of this and the work by Lang and White (39), 50-wk-old mice were used in the present study to represent a mature group. Mechanical performance was also assessed at 30 wk of age to represent a development group, in an attempt to not only assess the early onset of ageing, but also 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 hindlimb 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% O2-5% CO2) Krebs-Henseleit solution (mM: 118 NaCl, 4.75 KCl, 1.18 MgSO4, 24.8 NaHCO3, 1.18 KH2PO4, 10 glucose, and 2.54 CaCl2; pH 7.55 at room temperature prior to oxygenation). Aluminum foil T clips were wrapped around each tendon to minimize 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; aluminum 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 and diaphragm muscles were dissected from another animal of 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 was used to attach the muscle, via crocodile clips, to a force transducer (model UFI1, Pioden Controls) at one end and to a motor (model V201, Ling Dynamic Systems) at the opposing end. Position of the motor arm was detected via a linear variable-displacement transformer (model DF5G.0, Solartron Metrology, West Sussex, UK). In contrast to previous research examining the direct skeletal muscle ageing effect, where a much lower temperature was used (14, 23–25), the muscle was maintained in circulated oxygenated Krebs-Henseleit solution at a constant 37 ± 0.5°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 fiber itself but stimulated the muscle via the surrounding fluid. Muscle stimulation and length changes were controlled using custom-written software (CEC TestPoint, Measurement Computing, Norton, MA) via a digital-to-analog board (model KPCI3108, Keithley Instruments, Cleveland, OH) on a standard desktop personal computer.

Each muscle preparation was electrically stimulated while held at a constant length to produce a series of twitch responses. Muscle length and stimulus amplitude (14–18 V for EDL and 10–16 V for diaphragm; current fixed at 160 mA) were optimized to achieve maximal isometric twitch force. The muscle length that corresponded to max-
imal twitch force ($L_0$) was measured using an eyepiece graticule fitted to a microscope. Mean muscle fiber 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 fiber length exists for diaphragm muscle, the physical measurement was used as $L_0$. Maximal isometric tetanic force was measured by subjecting each muscle preparation to a 250-ms burst of electrical stimulation. The frequency of stimulation was further optimized in each muscle to yield maximal tetanic force; this was usually 200 Hz for EDL and 140 Hz 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-min rest period was imposed between each tetanus to allow sufficient time for the muscle to recover.

All EDL and diaphragm muscles were subjected to this process of isometric measures before the subsequent work-loop protocol. The muscle was held at the previously determined $L_0$, and the stimulation amplitude and frequency parameters that were optimized 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 $L_0$. Cycle frequencies of 10 and 7 Hz were used for EDL and diaphragm muscle, respectively; 10 Hz represents the cycle frequency that has been previously shown to elicit maximal power output in isolated mouse EDL (31), and 7 Hz represents 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 EDL and diaphragm muscle (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 TestPoint software. Data were sampled at a rate of 10 kHz; then a work loop was formed by plotting force vs. length, the area of which represents the net work done by the muscle at a rate of 10 kHz; then a work loop was formed by plotting force vs. 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 alteration of 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 49-ms burst duration was used to elicit maximal power output at 10-Hz cycle frequency. The burst duration to elicit maximal muscle power in diaphragm muscle was usually 55 ms. On occasions, it was necessary to alter the burst duration to adjust the number of stimuli given to maximize power output of an individual muscle preparation. This alteration in burst duration was determined by examination of the maximal work-loop power output and by interpretation of the work-loop shapes: too much activity of the muscle during relengthening will significantly distort the shape of the loop and reduce muscle power output by increasing the resistance of the muscle to stretch. Stimulation phase shifts of −2 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-min intervals until maximal muscle power output was achieved. The third work loop of each set of four typically produced the highest power and, therefore, was taken as the measure of muscle power output in all work-loop experiments. A 10-min rest interval was imposed between each set of four work loops to allow the muscle sufficient recovery time.

Sustained work-loop power. 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 elicited maximal power output. Data were recorded for each second loop until force was 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 min. Three measurements of maximal power output were made every 10 min and compared directly with maximal muscle power output prior to the repetitive muscle activation protocol.

The duration of the experimental protocol was 230 min, and control runs were performed regularly to monitor muscle performance over time. After 180 min, 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 that 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 were removed, leaving the muscle intact. Then the muscle was blotted on tissue paper to remove excess fluid and subsequently placed on an electronic balance (Mettler Toledo B204-S, Zurich, Switzerland) to determine wet mass. Immediately thereafter, 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 1,060 kg/m³ (47). Isometric stress was calculated as force ÷ mean muscle cross-sectional area. Muscle power output was normalized to muscle mass to express power as watts per kilogram.

Biochemical Analysis

Maximal activities of lactate dehydrogenase (LDH) and citrate synthase (CS), marker enzymes for maximal glycolytic ATP production potential and mitochondrial capacities, respectively, were measured. Furthermore, the maximal activity of the sarcoplasmic reticulum Ca2+-transporting ATPase (SERCA), an important regulator of Ca2+ resequestration into the sarcoplasmic reticulum (SR) and, therefore, muscle contraction-relaxation dynamics, was determined. Enzyme activities were determined according to published protocols (33, 59).

mRNA transcript content of ryanodine receptor (RYR) and fast and slow isoforms of SERCA and troponin was measured to determine if an age-related change in skeletal muscle mechanical performance was related to changes in the quantities of these important mediating proteins in Ca2+ release, force production, and Ca2+ 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) following the manufacturer’s instructions. RNA concentration and quality were verified using a spectrophotometer (Nanodrop Technologies, Thermo Scientific). An 800-ng sample of total RNA was treated with DNase I (Sigma) and reverse-transcribed using RNase H−Maloney’s murine leukemia virus reverse transcriptase (Biosearch, Bioline, Alexandria, NSW, Australia) and random hexamer primers (Bioline). Quantitative RT-PCR (model 7500, Applied Biosystems, Scoresby, VIC, Australia) was performed according to published protocols (69).

Prevalidated TaqMan gene expression assays (Applied Biosystems) were used according to the manufacturer’s instructions to quantify tropomin 1 (ttni1; assay ID: Mm00502426_m1), tropomin 2 (ttni2; assay ID: Mm00437157_g1), Ca2+-transporting ATPase 1 (atp2a1; assay ID: Mm01275320_m1), Ca2+-transporting ATPase 2 (atp2a2; assay ID: Mm01201431_m1), RYR1 (ryr1; assay ID: Mm01175211_m1), and elongation factor 1α (elf1α2; assay ID: Mm01514649_m1) expression. With ELF1α2 as the housekeeping gene. We used TaqMan gene expression master mix (Applied Biosystems) 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, and 60°C for 1 min.
Significant differences between age groups are indicated by common symbols.

Statistical Analysis of Data

Values are means ± SE. Datasets were analyzed by permutation analysis of variance (PERMANOVA; Primer 6 PRIMER -E, 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, which is preferable for relatively small datasets (22).

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 with prerepetitive activation values. 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 of recovery) with a one-way PERMANOVA.

Results were interpreted as significant when $P < 0.05$. In case of significant PERMANOVA results, we used post hoc pair-wise tests to compare specific age groups.

RESULTS

Body and Muscle Mass

Increasing age resulted in a significant increase in mean body mass [Fig. 1A; PERMANOVA degrees of freedom (df) = 3, 76, $F = 69.4, P < 0.01$]. Whole animal body mass increased significantly (Fig. 1A; pair-wise $t > 9, P < 0.01$) for each age group and was greatest at 50 wk of age (Fig. 1A; $t > 5.5$, pair-wise $P < 0.01$ in all cases). At 50 wk, individual body mass had increased above 70 g (Fig. 1A; group X) or stayed below 50 g (Fig. 1A; group Y), which is similar to the mean body mass at 30 wk of age. The distribution of the body masses between animals only permitted the analysis on the effects of skeletal muscle mechanical properties of diaphragm from 50-wk-old obese (Fig. 1A; group X) and lean (Fig. 1A; group 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/m² for groups X and Y, respectively) and work-loop power output (pair-wise $t < 2, P > 0.17$ in both cases), dynamic power output (expressed as W/kg muscle mass) was 25% greater for the lean than the obese group.

EDL muscle mass was significantly affected by age (Table 1, Fig. 1B; PERMANOVA $df = 3, 36, F = 56.3, P < 0.001$). Mean muscle mass was significantly lower at 3 wk than all other time points (Table 1, Fig. 1B; pair-wise $t > 7, P < 0.001$ in all cases). Maximum muscle mass was observed at 50 wk of age and was 29% and 13% greater than at 10 and 30 wk, respectively (Table 1, Fig. 1B; pair-wise $t > 4.2, P < 0.001$). Similar measures were not compared for diaphragm, as only a section

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

<table>
<thead>
<tr>
<th>Age</th>
<th>EDL</th>
<th>Diaphragm</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Twitch force, mN</td>
<td>3 wk</td>
</tr>
<tr>
<td></td>
<td>42 ± 3.9</td>
<td>100.3 ± 3.3</td>
</tr>
<tr>
<td></td>
<td>193.9 ± 14.3</td>
<td>337.3 ± 20.1</td>
</tr>
<tr>
<td>Muscle mass, mg</td>
<td>6.72 ± 0.5</td>
<td>12.44 ± 0.4</td>
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Values are means ± SE; $n = 10$ in each group. Data for extensor digitorum longus (EDL) represent whole muscle mass; data for diaphragm represent the section of the muscle used in evaluation of mechanical performance.

Fig. 1. Increasing age resulted in greater mean body mass (A) and extensor digitorum longus (EDL) muscle mass (B) of CD-1 mice. In A, data at 50 wk are further divided into mice with body mass >70 g (group X) and mice with body mass <50 g (group Y). Values are means ± SE; $n = 20$ (A) and 10 (B) in each group. Significant differences between age groups are indicated by common symbols.
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 df = 79, F = 7.9, P = 0.002). EDL twitch stress was greatest in 10-wk-old mice and was significantly lower at 3 wk (by 39%), 30 wk (by 20%), and 50 wk (by 27%) (Fig. 2A; pair-wise t > 2, P < 0.01 in all cases). EDL twitch stress was significantly higher at 30 wk than 3 wk (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-wk-old mice and was significantly lower at 30 (by 34%) and 50 wk of age (by 27%) (Fig. 2A; pair-wise t > 2.6, P < 0.02 in both cases). Mean diaphragm twitch stress had a tendency to be greater at 3 wk than 30 wk (Fig. 2A; pair-wise t = 2, P = 0.05).

**Maximal Isometric Tetanus Stress**

The mean maximal isometric tetanus stress for EDL (251 ± 17 kN/m²) and diaphragm (169 ± 10 kN/m²) muscle occurred at 10 wk of age and is consistent with values of 233–256 kN/m² for EDL (8, 31, 32) and 169 kN/m² 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 df = 79, F = 7.9, P = 0.001). For EDL and diaphragm, muscle maximal isometric stress occurred at 10 wk and was significantly lower at 3 wk (by 17% and 10%, respectively), 30 wk (by 18% and 28%, respectively), and 50 wk (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 wk compared with 3 wk (Fig. 2B; pair-wise t = 2.4, P = 0.011).

**Isometric Activation and Relaxation Times**

For EDL and diaphragm muscle, mean THPT and LSHR were significantly affected by age (Fig. 3; PERMANOVA df = 79, F > 6.2, P = 0.001 in both cases). In EDL, THPT was significantly longer at 3 wk (by up to 46%) than 10, 30, and 50 wk (Fig. 3A; pair-wise t > 4.47, P < 0.003 in all cases). LSHR at 10 wk of age and is consistent with values of 233–256 kN/m² for EDL (8, 31, 32) and 169 kN/m² 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.

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was significantly prolonged at 50 wk (by up to 32%) compared with 3, 10, and 30 wk (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 wk than 10 wk (Fig. 3A; pair-wise $t = 3.03, P = 0.012$) and had a tendency to be greater than at 3 wk (Fig. 3A; pair-wise $t = 1.91, P = 0.064$). LSHR was significantly greater at 50 wk than 3 and 10 wk (Fig. 3B; pair-wise $t > 1.9, P < 0.03$ in both cases).

**Work-Loop Power Output Normalized to Muscle Mass (Watts/kg)**

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

**Work-Loop Power Output Normalized to Whole Animal Body Mass (Watts/g)**

Mean muscle power output, normalized to body mass, was significantly affected by age for EDL muscle (Fig. 4B; PERMANOVA df = 3, 36, $F = 3.24, P < 0.032$). For EDL, mean maximal work-loop power output, when normalized to body mass, was highest at 10 wk and was significantly reduced at 3 wk (by 20%), 30 wk (by 19%), and 50 wk (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 EDL and diaphragm muscle (Fig. 5; PERMANOVA df = 3, 36, $F > 6.3, P < 0.002$ in both cases). For EDL muscle, the ability to sustain muscle power output over time was significantly reduced at 50 wk compared with all other time points (Fig. 5; pair-wise $t > 2.8, P < 0.001$ in both cases). Similarly, sustained muscle power
output of EDL was significantly reduced at 10 wk compared with 3 and 30 wk (Fig. 5; pair-wise $t > 2.4, P < 0.02$ in each case).

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

Typical work-loop shapes (Figs. 6 and 7) indicate an increased relaxation time during the relengthening phase over the course of the protocol in muscles in which power output declined more rapidly (10 and 50 wk in EDL and 10 wk in diaphragm), resulting in greater negative work and further contributing to the loss of net work (positive work during shortening – negative work during muscle relengthening) through repetitive activation.

**Recovery From Sustained Work-Loop Activation**

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

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

**Biochemical Analysis**

In EDL muscle, SERCA, CS, and LDH activity was significantly affected by age (Fig. 9; $df = 3, 26, F > 3.11, P < 0.03$ in each case). SERCA was significantly lower at 50 wk than 10 and 30 wk (Fig. 9; pair-wise $t = 2.36, P < 0.02$ in both cases) and had a tendency to be lower than at 3 wk (Fig. 9; pair-wise $t = 2.72, P < 0.008$). CS activity was significantly lower at 10 wk than at all other ages (Fig. 9; pair-wise $t > 4, P < 0.003$ in
all cases). CS activity was significantly lower at 3 wk than 30 and 50 wk (Fig. 9; pair-wise t > 4, P < 0.004 in both cases). LDH activity was significantly lower at 3 wk than 10 and 50 wk (Fig. 9; pair-wise t > 3.75, P < 0.005 in both cases) and had a tendency to be lower than at 30 wk (Fig. 9; pair-wise t = 2.03, P = 0.058).

For diaphragm muscle, LDH activity changed significantly with age (Fig. 9; PERMANOVA df = 3, 32, respectively; F = 3.42, P = 0.02). LDH activity was significantly lower at 3 wk than 10 and 30 wk (Fig. 9; pair-wise t > 2.28, P < 0.02) and had a tendency to be lower than at 50 wk of age (Fig. 9; t = 1.8, pair-wise P = 0.069). There were no significant differ-
ences in SERCA or CS activity (Fig. 9; PERMANOVA df = 3, 28, F = 1.6, P = 0.2).
mRNA for SERCA1, SERCA2, RYR1, Tnni1, and Tnni2 was not significantly different between age groups in EDL or diaphragm [Fig. 10; PERMANOVA df = 3, 20 (EDL) and 3, 22 (diaphragm), 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 begins at a relatively young age. More significantly, the limited change in the biochemical parameters suggests 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-wk-old mice produced the highest isometric stress, lowest activation and relaxation times, and highest power output and appeared to have a faster fiber type composition. In contrast to EDL, these parameters were already well developed in 3-wk-old diaphragm and, subsequently, may underline the importance of the physiological function of breathing compared with 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 wk 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 it has been demonstrated to increase in a fiber-specific manner (44, 60). It has been suggested that the SR content of skeletal muscle with a predominantly faster phenotype at maturity takes longer to develop and, as such, the optimized process excitation-contraction coupling occurs at a later age (44). This coincides with a prolonged time in the development of faster muscle fibers during growth, and Agbulut et al. (1) demonstrated that, at 21 days postgestation, type IIb myosin heavy chain represented 54% of the total proportion of EDL and increased to 87% at 90 days. As widely recognized, these fibers coincide with a greater normalized maximal force and power output and more rapid activation time due to enhanced contractile characteristics and

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Fig. 7. Effect of age on typical work-loop shapes of mouse diaphragm muscle during repetitive activation at 7-Hz cycle frequency for 3-, 10-, 30-, and 50-wk-old mice. Work loops 2 (0.29 s), 10 (1.43 s), and 18 (2.57 s) of the fatigue run are shown. Eccentric muscle activity in the relengthening phase of the work loop was increased in fatigued diaphragm from 10-wk-old mice compared with other age groups. Diaphragm muscles from 10-wk-old mice were associated with the poorest fatigue resistance.
The current findings indicate that muscular atrophy is not the sole contributor to reduced muscle performance during early ageing.

It has been suggested that significant muscle atrophy takes place after 10 wk of age. Brown and Hasser (15) suggested that this controversy may arise because of differences in the strain of rodents examined, the use of non-pathogen-free animals, and the age at which the animals are deemed to be aged. 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 because of differences in the strain of rodents examined, the use of non-pathogen-free animals, and the age at which the animals are 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 in the present study precede this. The current findings indicate that muscular atrophy is not the sole contributor to reduced muscle performance during early ageing.

A primary mechanism for the decline in mechanical performance in older age groups appears to be a shift toward a slower more oxidative fiber type (5, 7, 17), which results in a reduced potential to produce high force. Nevertheless, research suggests that older ageing evokes a reduction in oxidative capacity of the muscle largely attributed to a decline in mitochondrial function (17, 61), which is characterized by a reduction in oxidative enzymes such as CS (61). Interestingly, the given increase in CS in 50-wk-old EDL muscle in the present study contradicts this; therefore, it may be considered that an early age-related shift to slower fiber type may be effective in offsetting the decline in mitochondrial function due to the enhanced oxidative capacity of such phenotypes. Furthermore, as there were no concomitant changes in biochemical parameters of 50-wk-old diaphragm, this indicates that the early age-related reduction in mechanical performance may in part relate to mechanisms other than a change in muscle metabolic capacity.

The age-related reduction in muscular contractility may therefore relate to an increase in dysfunctional Ca²⁺-handling proteins, the most documented of which is the uncoupling of dihydropyridine-ryanodine receptors, resulting in a reduced Ca²⁺ availability at the contractile proteins (19, 40, 53, 58). Furthermore, the present findings support previous research indicating an age-induced inactivation of SERCA (40, 68). Interestingly, the reduction in SERCA activity does not correspond with a reduction in mRNA transcript content, which suggests that the buildup of dysfunctional SERCA proteins, rather than a loss in number, is more prevalent during early ageing. The reported age-related reduction in SERCA activity corresponds to the increase in relaxation time in EDL muscle in the present study (29, 40, 52).

When normalized to animal body mass, the reduction in muscle power output (W/g) from 10- to 50-wk-old EDL of ~ 22% was equal in magnitude to the loss of maximal force. Therefore, the animal is likely to move at a reduced pace and to 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. Muscle from 3-wk-old animals demonstrated the greatest ability to sustain power output, which was significantly reduced at 10 wk. This sus-
tained power output was significantly greater at 30 wk before a second wave of reduced sustained muscle power at 50 wk. 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 animal’s lifespan likely give rise to the equivocal in vivo and in vitro results from previous studies of the effect of ageing on muscular endurance (9, 11, 18, 30, 36, 41, 43, 55).

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

Previous isolated muscle research demonstrating increased (16, 55), decreased (73), and negligible (24) effects on the maintenance of muscle force with increasing age via repetitive isometric contractions is difficult to compare with the findings in the present study because of 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’s ability to sustain power output in work loops in response to changes in ability to produce force.

The age-related decline in muscle stress and ability to maintain power in 50-wk-old muscle may further relate to an age-induced increase in muscle collagen and fat, resulting in larger noncontractile 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-wk-old 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 relengthening phase of the work loop, as indicated by the work-loop shapes. If the muscle is active during relengthening, a greater proportion of negative work is conducted; 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-wk-old mice recovered to the greatest degree, and recovery at 30 wk 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 (23) concluded that, despite changes in maximal tetanic stress of EDL and soleus muscle from 22- to 24-mo-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 fiber type (65). Consequently, this may explain why EDL muscle from 3-wk-old mice recovered more quickly than EDL muscle 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 min to ~80–90%, as demonstrated in our previous work (28, 32).

Limitations and Practical Implications of the Study

The present research was 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 are 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 authors’ knowledge, the only study assessing the age- and sex-related changes in skeletal muscle contractility was conducted by Chan and Head (16). They demonstrated that the age-related decline in maximal absolute force and increase in isometric relaxation time of EDL from 22-mo-old mice appeared to affect females to a greater extent; however, there was 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 focus on the age- and sex-related decline in skeletal muscle contractility.

As previously suggested, ageing may promote a greater noncontractile mass, and as such the 1,060 kg/m³ value used in our calculations may overestimate muscle density in older animals and, as a result, underestimate cross-sectional area in muscles from the older age groups. This may result in stress being overestimated in older muscles; therefore, it is considered that the reduction in maximal stress may be greater than that portrayed in the present study. In addition, we recognize that previous studies examining the mechanical function of EDL have used slight variations in calculation of estimated mean muscle fiber 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 have been included (Table 1) to allow further comparison of maximal isometric force across the literature. Importantly, a change in the calculation of EDL fiber length will not affect the demonstrated trend and magnitude of effect shown in the present results.

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 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 force and power in the diaphragm than in the locomotory EDL muscle, which may warrant further research on the contribution of diaphragm muscle to the severity of respiratory symptoms in elderly patients (54, 56). Furthermore, the suggested age-related increase in central fatigue that occurs in endurance tasks may potentially magnify the ageing response in the present study when these results are related to in vivo performance (13, 20).

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

Conclusion

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

Perspectives and Significance

The evidence presented here offers the first 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 should be directed toward the con-
tribution of obesity and a sedentary lifestyle to the muscle ageing response.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

J.T., R.S.J., V.M.C., and F.S. are responsible for conception and design of the research; J.T. and A.G.L. performed the experiments; J.T., R.S.J., and A.G.L. analyzed the data; J.T., R.S.J., A.G.L., V.M.C., M.J.D., and F.S. interpreted the results of the experiments; J.T. and A.G.L. prepared the figures; J.T. and R.S.J. drafted the manuscript; J.T., R.S.J., V.M.C., M.J.D., and F.S. edited and revised the manuscript; J.T., R.S.J., A.G.L., V.M.C., M.J.D., and F.S. approved the final version of the manuscript.

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