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Age-Related Changes in Isolated Mammalian Muscle Function are Dependent on Sex, Muscle & Contractility Mode

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Running title: Muscle, force, power & fatigue in ageing male and female mice

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Key Points

- Our knowledge of age-related, muscle-specific and sex-specific changes in isolated skeletal muscle contractile function are derived from multiple studies of muscle ageing. A single, comprehensive assessment could improve our understanding of skeletal muscle ageing.
- After 10 weeks, an age-related increase in muscle mass is mirrored by an increase in absolute contractile performance, but a decrease in muscle quality, up to 52 weeks. Absolute function declined between 52 weeks and 78 weeks of age, alongside a reduction in female EDL mass, whilst male soleus contractile function was affected to the greatest extent.
- Age-related changes in contractile function occurred without a change myosin heavy chain isoform composition.
- Fatigue resistance was poorer for male diaphragm and female EDL.

- We report an increase and maintenance of absolute function, which is likely an adaptive response to an increased body mass, though the accelerated loss of function in old age will likely further impede locomotor function.

Abstract

The present study aimed to simultaneously examine the age-related, muscle-specific, sex-specific and contractile-mode-specific changes in isolated skeletal muscle function and morphology across multiple ages. Measurements of mammalian muscle morphology, isometric force and stress (force/cross-sectional area), absolute and normalised (power/muscle mass) work loop power across a range of contractile velocities, fatigue resistance, and myosin heavy chain (MHC) isoform concentration were collected from isolated mouse (CD-1) soleus, extensor digitorum longus (EDL) and diaphragm from male and female individuals aged 3-, 10-, 30-, 52-, and 78-wks. Ageing resulted in increased body mass, and soleus and EDL muscle mass, with atrophy only present for female EDL by 78-wks. Absolute force and power output increased up to 52-wks and to a higher level for males. The loss of isometric stress exceeded that of normalised power between 10 and 52-wks, though the loss of normalised power was greater than that of stress by 78-wks. Males had lower normalised power than females by 78-wks, with the greatest decline observed for male soleus. Ageing did not cause a shift towards slower contractile characteristics, with reduced fatigue resistance observed in male EDL and female diaphragm, but no change in slow or fast MHC isoform concentration. Skeletal muscle ageing is not a uniform process, where the age-related changes in function are sex, muscle and contractile-mode-specific, and is more complicated than what has been derived from smaller studies examining the muscle ageing response.

Introduction

Studies of musculoskeletal function in humans demonstrate an age-related reduction in force (Young *et al.*, 1984, 1985; Overend *et al.*, 1992; Goodpaster *et al.*, 2001) and power (Skelton *et al.*, 1994; Lauretani *et al.*, 2003; Pojednic *et al.*, 2012; Edwén *et al.*, 2014), with the loss of power exceeding that of strength (Metter *et al.*, 1997; Krivickas *et al.*, 2001; Raj *et al.*, 2010), and muscle mass (Suetta *et al.*, 2019). The loss of skeletal muscle function is also sex-specific, where the loss of absolute force and power occurs to a greater magnitude in males than females (Reed *et al.*, 1991; Skelton *et al.*, 1994; Lauretani *et al.*, 2003; Delmonico *et al.*, 2009; Edwén *et al.*, 2014). However, recent research in humans has shown that males produce greater absolute force in older age than females, but produce less force relative to muscle physiological cross-sectional area (CSA) (McPhee *et al.*, 2018) highlighting the disparity between measures quantifying the muscle ageing response. Ageing also results in a rapid deterioration in fatigue resistance when performing activities of daily living (Mueller-Schotte *et al.*, 2016), with an age-induced degradation in the coordinated system of peripheral and central components contributing to poor muscular fatigue resistance. When examining muscle fatigue specifically, however, some studies report a decline in muscular fatigue resistance (Davies *et al.*, 1986; Sunnerhagen *et al.*, 2000; Izquierdo *et al.*, 2001) that is greater in males than in females (Davies *et al.*, 1986; Hicks & McCartney, 1996), while others report no age-related or sex-based differences (Bilodeau *et al.*, 2001; Bazzucchi *et al.*, 2005). The overall age-related reduction in contractile performance is associated with poorer locomotor capabilities, greater all-cause mortality, and a lower quality of life (Bassey *et al.*, 1992; Horner *et al.*, 2011; Volaklis *et al.*, 2015; Tsekoura *et al.*, 2017; Celis-Morales *et al.*, 2018), with an age-related loss of muscular power having greater implications for independent physical functioning than the age-related loss of force (Raj *et al.*, 2010). A better understanding of the age-related changes in skeletal muscle morphology and contractile function can improve quality of life for the older population and reduce the ever-increasing financial burden on public health services in the treatment of age-related muscle disorders and their co-morbidities (Sousa *et al.*, 2016).

Human studies examining age-related changes in force, power output, and fatigue resistance have been valuable for furthering our understanding of the muscle ageing response. However, such measures typically assess absolute changes in force or power with

age or normalise function to body mass (Edwén *et al.*, 2014) or the physiological CSA of muscle groups such as the quadriceps (McGregor *et al.*, 2014; McPhee *et al.*, 2018). However, measurements of muscle quality (defined as force or power per unit of skeletal muscle mass) derived from measurements of whole muscle mass of specific muscle are more informative of the muscle ageing response (Tallis *et al.*, 2018). Measuring muscle quality is important as the age-related decline in muscle performance could be associated with a decline in muscle mass and/or the force production per unit size of skeletal muscle, known as sarcopenia (Rosenberg, 1989) and dynapenia (Clark & Manini, 2008) respectively. Older adults with high muscle quality can produce greater force for a smaller amount of muscle mass, which is advantageous in reducing the metabolic cost of maintaining muscle mass and reducing the contribution to overall bodily inertia (Russ & Lanza, 2011; Tallis *et al.*, 2018). Whilst some studies have successfully examined muscle quality *in viv*o (McGregor *et al.*, 2014; McPhee *et al.*, 2018), such techniques are unable to isolate the interference from the central nervous system and consider the muscle-specific nature of ageing (e.g. locomotor vs respiratory muscles). Age-related denervation of muscle fibres contributes to a reduction in contractile performance, thereby masking the factors that contribute to an age-related decline of function at the muscle level (Carlson, 2004). Furthermore, comparisons between studies examining fatigue resistance are difficult because of variations in experimental protocols (e.g. isometric vs. dynamic activation), the muscle/muscle groups examined, intensity, and a lack of studies directly measures the ability of muscles to sustain power output during repeated activity (Tallis *et al.*, 2014, 2018). Given these limitations, experimental approaches examining isolated muscle function have been used to further our understanding of the muscle ageing response.

An isolated muscle approach is valuable for measuring the maximal contractile response of specific muscles that are otherwise unachievable *in situ* and *in vivo*. Studies examining age-related changes in isolated skeletal muscle form and function typically compare muscle-specific changes in isolated mammalian locomotor muscles, usually the soleus (predominantly slow-twitch) or extensor digitorum longus (EDL; predominantly fast-twitch). Respiratory muscle function (diaphragm; mixed phenotype) is (Zhang & Kelsen, 1990; Criswell *et al.*, 1997, 2003; Cantillon & Bradford, 2000; Greising *et al.*, 2013; Elliott *et al.*, 2016), though some studies have performed simultaneous examinations of locomotor and respiratory muscle contractile function (Tallis *et al.*, 2014, 2017; Hill *et al.*, 2019). Rodents are

typically divided into two (young vs. old) (Pagala *et al.*, 1998; Chan & Head, 2010; Greising *et al.*, 2013) or three (young vs. adult vs. old) (Brooks & Faulkner, 1991; Graber *et al.*, 2013, 2015) age groups of single sexes, with only two studies to date that have compared sex-based differences in contractile function with increasing age in isolated mammalian locomotory (Chan & Head, 2010) and respiratory (Cantillon & Bradford, 2000) skeletal muscles. Studies of mammalian skeletal muscle primarily focus on measures of maximal isometric force, and use different animals species and strains, classify age groups differently, use differing contractility modes, and commonly set the test temperature at 25°C (Chan & Head, 2010; Graber *et al.*, 2015; Tallis *et al.*, 2018), thus making comparisons between the literature and to *in vivo* function difficult.

Whilst our understanding of maximal force-generating capacity has been valuable to our understanding of age-related changes in contractile function, power production is of greater importance for the completion of activities of daily living (Foldvari *et al.*, 2000). When compared to the majority of studies examining age-related changes in isolated muscle contractile function, there is a significant dearth in the literature examining age-related changes in power output using an isolated skeletal muscle model (Tallis *et al.*, 2014, 2017; Graber *et al.*, 2015; Hill *et al.*, 2018, 2019). Isotonic and isovelocity contractions have been used previously (Graber *et al.*, 2015), but typically overestimate power output otherwise derived from more physiologically relevant cyclical changes in muscle length and are therefore poor indicators of the *in vivo* contractility motions otherwise observed in power-producing skeletal muscles (James *et al.*, 1995, 1996). Usage of the work loop (WL) technique provides a more realistic replication of *in vivo* muscle function allowing the manipulation of length change velocity (cycle frequency; CF) and length change amplitude (strain) to more closely replicate that used *in vivo* (Josephson, 1985; James *et al.*, 1996). However, few studies have examined the age-related changes in isolated skeletal muscle contractile function using the WL technique, with our previous work using a fixed CF (Tallis *et al.*, 2014, 2017; Hill *et al.*, 2018), thus not accounting for age-related changes in contractile velocity.

Ageing is associated with a downward and leftward shift in the force-velocity relationship, mediated by a reduction muscle fascicle length (Thom *et al.*, 2007), and type II fibre atrophy (Raj *et al.*, 2010). Indeed, some studies using isolated mammalian skeletal muscles have reported a similar shift in shortening velocity of intact soleus fibres (Thompson & Brown, 1999), whole soleus & EDL (Graber *et al.*, 2015), and diaphragm segments (Zhang &

Kelsen, 1990) when derived from isovelocity contractions. Others report no change in contractile velocity with age (Brooks & Faulkner, 1988, 1994; Kim & Thompson, 2012). These studies using an isovelocity assessment to determine muscular power are limited in that this technique fails to consider the work required to re-lengthen the muscle, a necessity for the muscle to produce subsequent positive work (Josephson, 1985). The net work required to complete a shortening and lengthening cycle is accounted for in the WL technique. To date, no study has measured the relationship between power output and CF at multiple ages using the WL technique. Construction of a power output-cycle frequency (PO-CF) curve (James *et al.*, 2011; Hurst *et al.*, 2019; Hill *et al.*, 2019) can enhance our understanding of the muscle ageing response at the muscle level under more physiologically relevant length change cycles to determine whether there is a downward and leftward shift in the PO-CF curve with increasing age and whether the optimal CF required to elicit maximal power is altered by age.

Our current understanding of the muscle ageing has been pieced together by comparing the findings of multiple studies, where comparisons can be difficult to make due to differences in questions and experimental approaches. The current study is the first to simultaneously consider the age-related, sex-specific and muscle-specific (soleus, EDL and diaphragm) changes in animal and muscle morphology, myosin heavy chain (MHC) isoforms, isometric force, WL power output, and fatigue resistance using the WL, of male and female muscles at multiples ages (3 weeks to 78 weeks) from a single outbred mouse stock.

Methods

Animal Information

Ethical approval was provided by the Coventry University Ethics Committee (P27011; 05/01/2015) and all procedures involving the animals performed per the 1964 Declaration of Helsinki and its later amendments. White male and female CD-1 mice (Charles River, Harlow, UK) were purchased at age 3-9 weeks old and allowed to mature in-house at Coventry University in same-sex groups of 8-10 per cage in 12:12 hour light:dark cycles at a room temperature of ~20°C and 50% relative humidity. At 9-10 weeks of age, male mice were housed in groups of 3-4 to minimise fighting. This outbred mouse stock was chosen so that the genetic heterogeneity aligned more closely to humans (Rice & O'Brien, 1980). All mice were provided with *ad libitum* access to food (CRM(P); SDS/Dietex International Ltd,

Whitham, UK) and water. Mice aged 3 weeks were used within one week upon arrival at Coventry University, and all other animals were used ± 1 week of their target age. All other groups were allowed to mature to the following ages before experimentation: 10 weeks, 30 weeks (males only), 52 weeks and 78 weeks. Sample sizes for muscle mechanics measurements were n=26-30 mice for each age and sex. A 3-week age group, representing adolescence, was included to demonstrate the rate of ageing from a young age group to peak sexual maturity at 10 weeks of age. A 30-week old age group was used to measure the early ageing response in males, and data for this age group are already available for females (Tallis *et al.*, 2014). The 52-week-old age group represents a mature adult population, and 78-week-old mice represent an older population, where a 50% mortality rate for female CD-1 mice occurs at 78-80 weeks of age (Navarro *et al.*, 2002). In total, 232 muscle samples were analysed for mechanical performance.

Muscle Isolation and Preparation

At their target age, animals were sacrificed by cervical dislocation per the British Home Office Animals (Scientific Procedures) Act 1986, Schedule 1, and weighed to determine body mass. At room temperature (~22°C), the segment of animal that contained the target muscle was skinned, rapidly isolated (~20 minutes) and placed in chilled (~5°C), 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 (Tallis *et al.*, 2014; Hill *et al.*, 2018).

The soleus (68.3% type I, 31.7%, type IIa at 20 weeks for female CD-1 mice) (Messa *et al.*, 2019) or extensor digitorum longus (EDL; 2.6% type I, 22.3% type IIa, 26.7% IIx, 46.3% IIb, 2.1% type IIxb fibres at 20 weeks for female CD-1 mice) (Messa *et al.*, 2019) from the left hindlimb was initially rapidly (<5 minutes following sacrifice) excised and snap-frozen in liquid nitrogen for biochemical analyses. The reciprocal muscle from the contralateral hindlimb was excised for mechanical measurement. Aluminium foil T-clips were wrapped around the distal tendon of each locomotor muscle to avoid tendon slippage during muscular contractions (Tallis *et al.*, 2014). A small piece of bone was left at the proximal end of the muscle to allow for the muscle to be anchored in the muscle bath. For diaphragm (8.2% type I, 52.4% IIa, 34.2% type IIx, 5.2% type IIax at 20 weeks for female CD-1 mice) (Messa *et al.*, 2019), a ventral segment of the costal diaphragm was used to assess mechanical performance, whilst the

remainder was snap-frozen in liquid nitrogen for biochemistry. Aluminium foil T-clips were wrapped around the central tendon with two ribs at the opposing end of the diaphragm segment left intact to anchor the muscle in the bath. Previous studies from our lab have successfully examined the contractile properties of costal segments of diaphragm, with a drop of tissue viability comparable to that of soleus and EDL (Tallis *et al.*, 2014, 2018; Hill *et al.*, 2018, 2019; Hurst *et al.*, 2019).

Experimental Set-Up

The experimental set-up has been described in detail in previous publications (Tallis *et al.*, 2014; Hill *et al.*, 2018; Hurst *et al.*, 2019). In brief, the muscle bath consisted of a Perspex chamber filled via continuous circulation of oxygenated Krebs-Henseleit solution from a reservoir placed in a heater/cooler water bath (Grant LTD6G, Grant Instruments, Shepreth, UK) maintained at 37.0 ± 0.2°C, with temperature monitored via a digital thermometer (Checktemp C, Harvard Apparatus, Cambridge, UK). We used a test temperature of 37°C (James *et al.*, 2015), which is more physiologically and mechanically relevant (Caremani *et al.*, 2019), and has been rarely used in ageing research (Tallis *et al.*, 2014; Hill *et al.*, 2018, 2019). The proximal end of the muscle was attached with crocodile clips to a force transducer (UF1, Pioden Controls Ltd, Henwood Ashford, UK) and a motor arm (V201, Ling Dynamic Systems, Royston, UK) at the distal end. The motor arm position was detected via a Linear Variable Displacement Transducer (DFG5.0, Solartron Metrology, Bognor Regis, UK). Muscle activation was achieved through electrical stimulation of the surrounding solution via parallel platinum electrodes, with electrical currents provided by a benchtop power supply (PL320, Thurlby Thandar Instruments, Huntingdon, UK). Visualisation of changes in force and length was provided by a storage oscilloscope (2211, Tektronix, Marlow, UK). Stimulation and length change parameters were manually manipulated via custom-written PC software (CEC Testpoint, Measurement Computing, Norton, MA) via a D/A board (KPCI3108, Keithley Instruments, Cleveland OH). Data were sampled at a rate of 10kHz, and WLs were plotted as the product of force and length, with the area within the WL representing the net work done by the muscle during a single length-change cycle (Josephson, 1985).

Once in position, each muscle was allowed to stabilise for 10-minutes before the commencement of the experimental protocol. The experimental protocol consisted of

isometric twitch and tetanus activations, work loop activations at a variety of cycle frequencies, a fatigue protocol and a recovery protocol as described below.

Isometric Activations

All muscles underwent a series of twitch activations to optimise mean muscle fibre length (L_0) and stimulation parameters. The physical length and stimulation voltage (14-20V for EDL, 12-17V for soleus, and 12-18V for diaphragm) were altered until maximal isometric twitch force was achieved. Stimulation current (160mA) and pulse width (1.2ms) were fixed at values that generated maximal twitch force in our previous studies (Tallis *et al.*, 2014; Hill *et al.*, 2018; Hurst *et al.*, 2019). The stimulation voltage required to achieve peak twitch force for all muscles was unaffected by age or sex (ANOVA; P>0.67 for both). Once peak twitch force was obtained, whole muscle length was measured using an eyepiece graticule fitted to a microscope, and L_0 calculated as 85% of muscle length for soleus and 75% of muscle length for EDL (James *et al.*, 1995). No estimation of mean muscle fibre length exists for diaphragm, so physical muscle length was used instead (Tallis *et al.*, 2014, 2017; Hurst *et al.*, 2019; Hill *et al.*, 2019). The maximal tetanic force was measured by subjecting the EDL and diaphragm to a 250ms burst of electrical stimulation and the soleus to a 350ms burst. The frequency at which the stimulations were provided was altered until peak tetanic force was achieved; this was typically 120-140Hz for soleus, 120-150Hz for diaphragm and 200-220Hz for EDL. The duration of isometric muscle activation and relaxation was measured as the time from the first stimulus to half-peak tetanus (THPT), and the time from the last stimulus to half-tetanus relaxation (LSHR), respectively. Five minutes of rest were imposed between each tetanic stimulation to allow for sufficient recovery. Once maximal isometric force was achieved, a further five minutes rest was imposed before commencing the WL protocol. This protocol for measuring isometric force and L_0 has been used in our previous work (Tallis *et al.*, 2014; Hill *et al.*, 2018, 2019; Hurst *et al.*, 2019).

Work Loop Protocol

Each muscle was held at the previously determined L_0 and the stimulation voltage and frequency that resulted in maximal tetanic force were implemented. Sinusoidal length changes were implemented to examine power output across a range of contractile velocities

(cycle frequencies; CF). Initially, a CF of 10Hz for EDL, 7Hz for diaphragm and 5Hz for soleus was used as these CF's typically elicited maximal power output in previous research for using these locomotory (Tallis *et al.*, 2014; Hill *et al.*, 2019) and respiratory (Tallis *et al.*, 2014; Hurst *et al.*, 2019) skeletal muscles. An initial strain of 0.10 (±5% relative length change from L_0) was used for all muscles, with phasic bursts of electrical stimulation provided per shortening segment of the sine wave for durations of 50ms, 55ms and 65ms to the EDL, diaphragm and soleus respectively. A phase shift of -2ms, -5ms and -10ms was used for EDL, diaphragm and soleus, respectively, to ensure maximal work. These phase shifts meant that the electrical stimulation started at these specific times before the muscle reached its maximal length. A set of four WL's was performed every 5-minutes, where net work of the third WL was recorded because this typically elicited peak net work and was therefore used as the measure of muscle power output for each experiment. Net work was determined across a range of CF's to produce a power output-cycle frequency curve (PO-CF) (James *et al.*, 2011; Hurst *et al.*, 2019; Hill *et al.*, 2019). CF's ranged from 4-16Hz for EDL, 3-12Hz for diaphragm and 2-10Hz for soleus and the order of CF was randomised, except for 10Hz for EDL, 7Hz for diaphragm, and 5Hz for soleus which were always examined first. Strain and stimulus burst duration were altered via the Testpoint software at each CF until peak net work was achieved. Generally, as CF increased, strain and stimulus burst duration decreased and vice versa. Preliminary work indicated that phase had a limited effect on net work at each CF and was kept constant for each CF and muscle. Whilst burst duration to elicit maximal power did not change with age, 52 week and 78-week-old muscles tended to require a smaller strain at each CF. For example, a ±5% length change from L_0 (strain 0.10) at 5Hz was usually optimal for 10-week-old muscles, but occasionally a ±4% (strain 0.08) length change from L_0 was optimal for 52 week and 78-week-old muscles.

Control sets of WLs (at 10Hz, 7Hz and 5Hz for EDL, diaphragm, and soleus, respectively) were performed after every three CF's and following the final CF to monitor the possible deterioration of power over time (Barclay, 2005). Power declined by an average of 14 ± 6% over ~160 minutes for all muscles as observed previously (Tallis *et al.*, 2014, 2017). This approach allowed for correction of net work for other CF's relative to the control WL's. Following the final control stimulation, each muscle underwent 10-minutes of rest before the fatigue protocol.

Fatigue Resistance and Recovery

To determine the fatigue resistance of each muscle, 50 consecutive WL cycles were imposed on each muscle using the optimised, muscle-specific, strain and stimulation parameters for the initial CF's used at the start of each experiment (i.e. 5Hz, 7Hz and 10Hz for soleus, diaphragm and EDL respectively). The net work of the third loop of every second set of WL's was recorded and plotted relative to the pre-fatigue maximal power output at that CF (Tallis *et al.*, 2014; Hill *et al.*, 2018, 2019; Hurst *et al.*, 2019). The time taken for power to drop below 50% of the pre-fatigue maximum power was recorded.

The ability of each muscle to recover from the fatigue run was monitored for 30-minutes immediately following the fatigue run. Every 10-minutes, one set of WL cycles was delivered to each muscle using the same stimulation parameters used to fatigue the muscle, and net work was recorded. Net work was directly compared to the pre-fatigue maximal power output and provided a percentage of the maximal power output. After 30 minutes of recovery, power output relative to the pre-fatigue maximal power output recovered to (mean ± S.D.) 93.9 ± 6.5% for soleus, 55.0 ± 18.1% for EDL and 90.0 ± 7.2% for diaphragm when averaged for each sex and age (Figure S1 A-F). These values are in keeping with our previous work (Tallis *et al.*, 2014; Hill *et al.*, 2018).

At the end of the experiment, the muscle was detached from the rig, tendons and excessive fluid removed, then weighed on an electronic balance (TL-64, Denver Instrument Company, Arvada, CO, USA) to determine wet muscle mass. Mean muscle CSA (m^2) was calculated as muscle mass (kg) divided by the product of muscle length (m) and an assumed muscle density of 1060$kg.m^{-3}$ (Méndez & Keys, 1960). Isometric stress ($kN.m^2$; used as an indicator of muscle quality) was calculated as peak tetanic force divided by mean muscle CSA. Absolute power (Watts) was calculated as the product of net work and CF and was normalised to muscle mass ($W.kg^{-1}$ muscle mass; used as a second indicator of muscle quality) at each CF. As per our previous work (Tallis *et al.*, 2017; Hill *et al.*, 2018, 2019; Hurst *et al.*, 2019), measures of muscle mass, muscle length, and absolute force and power are not reported for the diaphragm due to the slight variations in muscle size as a result of the dissection process, despite attempting to excise the same segment of the diaphragm from each animal (Lynch *et al.*, 1997). Therefore, only isometric stress and normalised power are reported for the diaphragm.

Biochemical Analyses

Fast and slow MHC expression was measured to examine changes in fibre-type composition. Proteins were extracted in RIPA buffer (in mM: Tris·Cl 20, NaCl 150, EDTA 1, EGTA 1, NP-40 1%, sodium-deoxycholate 1%, pH 7.5) with the addition of a protease and phosphatase inhibitor cocktail (Roche, Sydney, NSW, Australia). Protein concentrations were determined by capillary electrophoresis in a "Wes" Simple Western system (Protein Simple, Santa Clara, CA), according to the manufacturer's instructions. All antibodies were from the University of Iowa Developmental Studies Hybridoma Bank, and we determined concentrations of total fast (antibody #F59) and slow (BF32) skeletal MHCs, and α-tubulin (12G10) as an internal control (Lee *et al.*, 2012). All antibody and protein concentrations were optimized following the manufacturer's recommendations. We interspersed samples from different treatments on the same plate to avoid order effects.

Statistical Analyses

All data are presented as mean ± S.D., and the level of significance was set at $P<0.05$ for all analyses. Tests for normality were checked with the Shapiro-Wilk test and sphericity with the Mauchly test in SPSS (SPPS, v26.0, Chicago, IL, USA). Analysis of variance (ANOVA) tests was conducted using SPSS using a two-factor ANOVA with sex and age as the fixed factors. Dependent variables included measures of animal and muscle morphology (body mass, soleus and EDL muscle mass, muscle length, muscle CSA and slow/fast MHC isoform content), isometric properties (peak tetanus force, tetanus stress, THTP, LSHR) and time to 50% of the pre-fatigue maximum power for each muscle. A three-factor ANOVA was used to determine significant changes in soleus and EDL absolute power output, power output normalised to body mass, and power output normalised to muscle mass for all muscles. Absolute and normalised power were used as dependent variables, with sex, age and CF used as the fixed factors. For all two-factor and three-factor ANOVA's, differences between sex and age groups were examined by single-factor ANOVA's when an interaction was observed, to investigate main effects. Recovery of power was examined using repeated measures three-factor ANOVA's to determine whether sex, age and time affected the recovery of WL power 30 minutes following the fatigue protocol. For all ANOVA's, Tukey's *post hoc* analyses were used when significant differences were present for the main effects. Individual P-values from

the ANOVA's and post-hoc analyses are reported in supplementary tables S1-S3 for sex-based differences (Table S1) and age-related differences for females (Table S2) and males (Table S3).

Regression analyses were performed in GraphPad Prism for Windows (v8.2.1, GraphPad Software, La Jolla, California, USA) to examine the association between absolute power output (soleus & EDL) and power output normalised to muscle mass (all muscles) compared to animal body mass to determine whether larger animals generated greater absolute power to overcome bodily inertia at different ages for each sex and whether muscle quality of larger animals was poorer with increasing age. All animals for each analysis were pooled by age, but not sex.

Percentage changes from the maximal mean value for each of the aforementioned variables were calculated and presented in Table 1, where maximum represents the age at which a particular variable was greater than all other ages. In addition, percentage differences between males and females at a given age for each variable were calculated and presented in Table 1. To calculate the magnitude of differences in absolute and normalised power output for age and sex, the percentage decline in power output for each CF was combined and an average calculated to provide mean percentage decline for each muscle at each age group. This was done to provide a clear visual representation in trends of age-related and sex-specific changes in morphology and contractile function.

Effect size (ES) was calculated to determine the magnitude of the effect of sex on morphological or contractility parameters at a given age (Table S1). Cohen's *d* was calculated and corrected for bias using Hedge's *g* due to the small sample sizes for each experimental group (Hedges, 1981). The thresholds for determining the standardised ES's were determined as: <0.2 trivial; ≥0.2, <0.6 small; ≥0.6, <1.2 moderate; ≥1.2 large (Hopkins *et al.*, 2009).

The truncated product method (Zaykin *et al.*, 2002) was used to analyse the distribution of *P*-values to provide a *P*-value for each group of multiple hypothesis tests to assess whether these values were biased via multiple hypothesis testing. The truncated product method *P*-value was <0.0001, demonstrating that the results were not biased by multiple hypothesis testing.

Results

Animal and Muscle Morphology

Ageing resulted in a significant change in mean animal body mass, and soleus and EDL muscle mass and CSA ($P<0.002$; Figure 1 A-E). Males had significantly greater body mass compared to females ($P<0.001$) and had greater muscle mass and CSA for soleus and EDL ($P<0.003$ for all). Body mass was higher for males than females at 3 weeks and 10 weeks (Figure 1A; $P<0.0001$, $ES>1.60$ for both), and soleus muscle mass and CSA were greater for males at 3 weeks (Figure 1 B&C; $P<0.01$, $ES>1.45$ for both). EDL muscle mass and CSA were greater for males than females at 3 weeks, 52 weeks and 78 weeks (Figure 1 D&E; $P<0.002$, $ES>1.44$ for all). No effect for sex was observed for optimal muscle length (L_0) (Table 1; $P>0.09$, $ES<1.23$), though there was a large ES for L_0 for 3-week EDL (Table S1). Apart from L_0, each morphological variable increased from 3 weeks and 10 weeks and plateaued between 30 weeks and 78 weeks (Figure 1 A-E; Table 1). However, EDL muscle mass and CSA for females declined by 15% and 16% respectively between 52 weeks and 78 weeks (Figure 1 D&E; $P<0.005$ for both), indicating a significant age-related muscular atrophy not observed for other muscles. Soleus and EDL L_0 increased significantly from 3 weeks to 10 weeks but thereafter remained unchanged with age, with no differences between males and females (Table 1; $P>0.09$ for both muscles).

Tetanus Force and Tetanus Stress

Maximal tetanus force and tetanus stress (force produced per cross-sectional area of muscle, a measure of muscle quality) were significantly affected by age for all muscles of both sexes (Figure 2 A-F; $P<0.001$). A significant effect of sex was observed for EDL tetanus stress ($P=0.016$), where female tetanus stress was significantly greater at 78 weeks of age compared to males (Figure 2 D; $P<0.05$, $ES=1.02$). However, no further significant sex-based differences were observed for any other muscles or measures (Figure 2 A, B, C&E; $P>0.11$, $ES<1.83$). Large ES's were observed for 3-week soleus force and 78-week soleus stress (Table S1).

Tetanus force for male and female soleus and EDL increased from 3 weeks of age, peaking at 10 weeks (female EDL), 30 weeks (male soleus), 52 weeks (male EDL) and 78 weeks of age (female soleus) (Figure 2 A&C). By 78 weeks of age, tetanus force declined significantly

in soleus in males only, while EDL force and stress declined significantly in both sexes. Female soleus tetanus force did not change significantly from 10 weeks of age onwards (Figure 2 A&C; Table 1). Tetanus stress increased significantly from 3 weeks to 10 weeks in all muscles, where peak tetanic stress occurred. From 10 weeks of age, there was a significant decline in stress in all muscles of each sex up to 52 weeks of age. In males, a significant decline in tetanus stress was observed between 10 weeks and 30 weeks of age in soleus ($P=0.02$), but not in EDL or diaphragm ($P>0.21$ in each case; Table S2 & S3). Between 52 weeks and 78 weeks of age, there was no further significant decline in tetanus stress in any muscles of either sex ($P>0.23$ in all cases; Table S2 & S3).

Activation and Relaxation Times

Time to half-peak tetanus (THPT) was significantly affected by age in soleus (Figure 3A; $P=0.001$), but not in EDL or diaphragm (Figure 3 B&C; $P>0.13$ for both). THPT was significantly shorter in males in soleus and EDL (Figure 3 A&C; $P<0.004$) but not in diaphragm (Figure 3 E; $P=0.21$). Male THPT was faster than females at 10 weeks and 78 weeks in soleus (Figure 3 A; $P<0.05$, $ES>0.82$ for both), and faster than females at 3 weeks and 10 weeks in EDL (Figure 3 C; $P<0.03$, $ES>1.41$ for both). Male THPT in soleus remained unchanged between 3 and 52 weeks of age, increasing significantly at 78 weeks compared to 10 weeks ($P=0.04$), while female soleus THPT was significantly slower at 52 and 78 weeks compared with 3 weeks (Figure 3 A; $P<0.04$).

Last stimulus to half relaxation (LSHR) was significantly affected by age in all skeletal muscles (Figure 3 B, D&F; $P<0.04$ for all). LSHR time in male soleus was shorter than in females (Figure 3 B; $P=0.008$), and LSHR was significantly shorter at 3 weeks of age in soleus (Figure 3B; $P=0.045$, $ES=0.95$). LSHR in EDL and diaphragm, however, were not different between sexes (Figure 3 D&F; $P>0.72$). While there was an effect of age on LSHR times in soleus, post-hoc analyses were not powerful enough to detect significant age-associated differences in either sex (Table S2 & S3; $P>0.06$ for both sexes). Male and female EDL LSHR times remained largely unchanged between 3 weeks and 52 weeks of age but were significantly longer at 78 weeks compared to 3- and 10-week old EDL ($P<0.004$). No significant differences were observed between 52 and 78 weeks of age. Female diaphragm THPT increased significantly from 3 to 10 weeks, remained stable up to 52 weeks, before increasing at 78 weeks when

compared with 3 and 10-week female diaphragm (Figure 3 F). However, no age-related changes in LSHR time were observed in male diaphragm (Figure 3 F; P=0.32).

Absolute Power Output and Normalised Power Output

Absolute power output was significantly affected by age and sex in for soleus and EDL (Figure 4 A-D; P<0.001). In male soleus, power output was significantly greater than in females at 3 weeks, 10 weeks, and 52 weeks (Figure 4 A&B; P<0.001, ES>0.67 in all cases), but not at 78 weeks (P=0.96, ES=0.01). In male EDL, absolute power output was significantly greater than in female EDL at 3 weeks, 52 weeks and 78 weeks (Figure 4 C&D; P<0.001 ES>0.67 for all), but mean absolute power in females was greater than in males at 10 weeks (P<0.001; 57). Absolute power output increased with age and peaked at 30 weeks of age for male soleus while absolute power output peaked at 52 weeks in female soleus and EDL of both sexes. At 78 weeks of age, power output declined significantly in both soleus and EDL of both sexes (P<0.001), with the greatest decline in absolute power occurring in male soleus (Table 1). CF significantly affected power output (Figure 4 A-D; P<0.001), although no sex*CF interaction was found in soleus or EDL (Figure 4 A-D; P>0.11) indicting that sex did not influence the CF required to achieve peak power. An age*CF interaction was observed in soleus (Figure 4 A&B; P<0.001) but not in EDL (Figure 4 C&D; P>0.16), nor was a sex*age*CF interaction observed in soleus or EDL (Figure 5 A-D; P>0.86). The age*CF effect in soleus is due to absolute power at 10Hz being similar at all ages (P>0.08 in all cases) as opposed to a change in the optimal CF for maximal power output.

Changes in power output normalised to body mass largely followed a similar pattern to age-related and sex-specific changes in absolute power output for both soleus and EDL muscles (Figure 5 A-D & Table 1). However, female soleus generated more power relative to body mass at 78 weeks than male soleus (Figure 5 A&B; P=0.05, ES=0.30). However, no sex-specific differences were observed between males and females for EDL power output normalised to body mass (Figure 5 C&D, P=0.54, ES<0.87).

Power output normalised to muscle mass was affected by age for soleus, EDL and diaphragm (Figure 6 A-F; P<0.001). Normalised power output peaked at 10 weeks of age in all muscles, except for male EDL that peaked at 52 weeks of age. From the age at which peak normalised power output occurred, further increases in age resulted in significant declines in normalised power output in all muscles of each sex (P<0.001). In addition, there were

significant effects of sex on normalised power output in all skeletal muscles (Figure 6 A-F; P<0.02 in all cases). A sex*age interaction was observed in all skeletal muscles (Figure 6 A-F; P<0.001). In male soleus, normalised power was greater than in females at 3 weeks, 10 weeks and 52 weeks (P<0.02, ES>0.23 for all) whilst normalised soleus power in females was greater than in males at 78 weeks (Figure 6 A&B; P=0.001, ES=0.40). Female EDL normalised power output was significantly greater at 10 weeks and 78 weeks than males (P<0.001, ES>0.21 for both), but no differences were found at 3 weeks and 52 weeks (Figure 6 C&D; P>0.59, ES<0.07). Male diaphragm normalised power was significantly greater than in females at 3 weeks only (P=0.016, ES=0.40), whilst female diaphragm normalised power was significantly greater at 10 weeks and 78 weeks (P<0.04, ES>0.34 for both) but no difference observed at 52 weeks (Figure 6 E&F; P=0.06, ES=0.23). CF significantly affected power output for all muscles (Figure 6 A-F; P<0.001 for all). A sex*CF interaction was not observed for any skeletal muscles (Figure 6 A-F; P>0.15). An age*CF interaction was found in soleus (Figure 6 A&B; P<0.001) but not EDL or diaphragm (Figure 6 C-F; P>0.46), nor was a sex*age*CF interaction observed in any muscle (Figure 6 A-F; P>0.43). The age*CF interaction was due to soleus normalised power at 10Hz being similar at all ages, as opposed to a directional shift in the PO-CF curve determining where peak power occurred.

Regression analysis of the relationship between absolute power output and animal body mass showed a significantly positive relationship for soleus (Figure S2 A&B; r^2>0.435 for males; r^2>0.551 for females; P<0.0001 for both) and EDL (Figure S2 C&D; r^2>0.327 for males, r^2>0.329 for females; P<0.0004 for both). No association between animal body mass and power output normalised to muscle mass were observed in any muscle of either sex (Figure S3 A-F; r^2<0.069 for all; P>0.12 for all).

Fatigue Resistance

Muscle power output during repeated WL's was significantly affected by age in all muscles (Figure 7 A-F; P<0.001). Female soleus was more fatigue-resistant than that of males at 10 weeks and 78 weeks (Figure 8 A&B; P<0.05, ES>0.88 for both), but there were no significant differences between sexes in EDL or diaphragm (Figure 7 C-F; P>0.20, ES<1.17 for both).

3-week old male and female soleus were significantly more fatigue-resistant than at any other age (Figure 7 A&B; P<0.04 for both sexes), with no difference in fatigue resistance

from 10 weeks to 78 weeks for both sexes (P>0.56 for all). 3-week and 10-week-old female EDL was more fatigue-resistant than 52-week-old female EDL (Figure 7D; P<0.005 for both). For male EDL, fatigue resistance was greatest at 3 weeks of age compared to all other ages (Figure 7 C; P<0.001 for all). 10-week-old male EDL was more fatigue resistant than 52-week-old EDL, but not compared with 30-week and 78-week EDL (Figure 7 C, Table S3). 30-week-old male EDL was not more fatigue resistant than 52-week-old EDL and 78-week-old EDL, with no difference between 52 weeks and 78-week-old male EDL.

Male diaphragm fatigue resistance was not significantly affected by increasing age (Figure 7 E; P=0.22), whereas female diaphragm was more resistant to fatigue at 3 weeks than all other ages (Figure 7 F; P<0.01 for all). At 10 weeks, female diaphragm had greater fatigue resistance than at 52 and 78 weeks (Figure 7 F: P<0.05 for both) although no difference was found from 52 weeks to 78 weeks (Figure 7 F; P=0.36).

Typical work loop shapes (Figure S4-6) showed little alteration in the shape of the work loop for soleus during the time course of the fatigue with increasing age (Figure S4 A-H), although 3-week old female soleus produced substantially less net work (positive work during active shortening minus negative work during passive muscle lengthening and relengthening) than age-matched male soleus, as did 3-week old female EDL (Figure S5 A&B). However, 3-week-old female soleus WL shapes did not change substantially for the first 18 WL's, whereas 3-week-old male soleus had slightly more pronounced negative work. Negative work during relengthening was also far greater for 78-week-old male than female at loop 2, though this had little effect on absolute power output and fatigue resistance. For male EDL, the ability to maintain net work diminished between 10 weeks and 52 weeks of age, where net work was poorest compared to all other ages (Figure S5 E). An increase in fatigue resistance by 78 weeks for male EDL was associated with less negative work during relengthening and greater net work by loop 18. Female EDL, however, was able to better maintain net work during fatigue with increasing age, with less negative work during relengthening (Figure S5 A-F), with far less negative work during muscle lengthening by loop 18 at 52 weeks of age when compared male EDL. Moreover, 78-week-old female EDL was better able to maintain force during muscle shortening than EDL by loop 18 (Figure S5 H). The age-related decline in net work with fatigue was poorly maintained in females than males, where net work was generally greater for males

and negative work during muscle relengthening less amplified than that for females (Figure S6 A-H).

Analyses of MHC Isoforms

There was a significant sex-based difference in slow/fast MHC isoforms for diaphragm, (Figure 8 I; P=0.048) though post-hoc analyses failed to find any further sex-based differences at 10 weeks and 78 weeks of age (Table S1, P>0.13, ES<0.86). There were no further significant differences between males and females (Figure 8 A-H; P>0.05) or age-related changes (Figure 8 A-I; P>0.11) in MHC isoforms for all skeletal muscles, nor were any significant sex*age interactions observed (Figure 8 A-I; P>0.13).

Discussion

The present study shows that ageing is a highly complicated, non-uniform process, where the rate of loss of function, and some instances a limited change in function, is dependent on the animal sex, the modality of muscle activation, the muscle examined, and normalisation of muscle function to muscle size, which was only possible by simultaneously performing these measures for both males and female across multiple ages. Our findings, which we believe is the largest study of skeletal muscle ageing at the isolated muscle level to date, demonstrate that by using this experimental approach to examine the muscle ageing response, the effects of ageing on skeletal muscle may be more complicated than what we have been able to previously derive from smaller, more focused studies of the muscle ageing response in rodents.

We uniquely demonstrate that ageing generally resulted in increased absolute force and power up to 52 weeks of age, but led to a continual decrease in estimates of muscle quality (force normalised to muscle CSA and power output normalised to muscle mass) from peak maturity (i.e. 10 weeks) onwards, where the loss of muscle quality affected male soleus to the greatest extent. Only by the oldest age were significant declines in absolute force and power observed, and to a similar magnitude as muscle quality. Changes in muscle function occurred without prevalent atrophy as demonstrated by an age-related increase in muscle mass, with significant atrophy only evident by 78 weeks of age for female EDL. Additionally, no age-related alterations in MHC isoforms were observed at 10 weeks and 78 weeks of age,

indicating a change in fibre type towards a more oxidative capacity is not responsible for alterations in locomotor and respiratory muscle quality.

The current data are the first to show that in early ageing, the loss of isometric stress occurs faster than that of normalised power in early ageing as with our previous work (Tallis *et al.*, 2014), but this relationship is reversed by 78 weeks of age. Moreover, ageing causes an age-related increase in absolute force and power in early age despite a loss of muscle quality, though declined to a similar magnitude as that of isometric stress and power output normalised to muscle mass by 78 weeks of age. The rate of the decline in isometric stress and power normalised to muscle mass was greater in males than for females when comparing the age at which muscle quality was best and 78 weeks of age. Finally, ageing affected fatigue resistance for male EDL and female diaphragm only, with no further age-related declines in fatigue resistance observed for soleus.

Age-Related and Sex-Based Differences in Animal Morphology

Body mass (Figure 1 A) and soleus and EDL muscle mass and muscle CSA were significantly greater in males than females at 3-weeks (Figure 1 B-E). Maturation in mammals accounts for a significant portion of body and muscle growth and development, where factors such as elevated sexual hormone release, particularly testosterone in males, provide reasoning for the greater development of muscle mass in male compared to female rodents (Joubert *et al.*, 1994). In adolescence in humans, males experience a ten-fold increase in circulating testosterone levels leading to increased muscle mass and body mass, whilst increased oestrogen in females during maturation is associated with a markedly smaller increase in body mass, but greater body fat deposition in humans (Beunen & Malina, 1988; Round *et al.*, 1999).

Further ageing from peak maturity to old age caused a significant increase in animal body mass, muscle mass and muscle CSA for both sexes up to 52 weeks of ages, though significant atrophy was observed for female EDL by 78 weeks of age. Previous data for female CD-1 mice report no age-related reduction in whole muscle mass of the soleus or EDL when comparing 10-week-old mice to 52-week (Tallis *et al.*, 2014) and 78-week-old mice (Hill *et al.*, 2018). Histological data presented by Messa *et al*. (2019), report a significant reduction in the percentage of type IIa fibres for soleus, and an overall reduction in fibre CSA for the EDL by 79 weeks of age for female CD-1 mice. As such, the increase in total muscle mass in the

present study may be partially attributable to age-related ectopic storage of fat and collagen (Kragstrup *et al.*, 2011), which could be to a greater magnitude for male EDL than female EDL due to the greater muscle mass and CSA by 52 and 78 weeks (Figure 1 D&E). However, this does not appear to be the case in older adults, where males and females exhibited similar levels of intramuscular fat accumulation in the quadriceps (Delmonico *et al.*, 2009). Whilst we did not analyse muscle morphology for the diaphragm, Messa & colleagues (2019) reported fibre hypertrophy, as demonstrated by increased total fibre CSA, for the diaphragm by 79 weeks of age. Unaltered fibre size and composition have been reported in very old (27 months) C57BL/6 x 129 mice when compared to old (24 months) mice, thus suggesting a critical threshold for fibre atrophy, which could be related to the central role of the diaphragm in respiration (Vang *et al.*, 2020).

Changes in Absolute and Normalised Force & Power Output

3-week-old male locomotor muscles generated greater absolute power output and power output normalised to muscle mass than females (Figure 4 A-D), with no differences in absolute force or stress (Figure 2 A-D), whilst male diaphragm produced greater power output normalised to muscle mass than females (Figure 2E). Differences between sexes and age are largely attributed to fibre type and sexual maturation as previously described.

Previous work examining age-related changes in absolute force and isometric stress report that the decline in isometric stress typically exceeds that of force (Phillips *et al.*, 1991; Moran *et al.*, 2005; Chan & Head, 2010; Tallis *et al.*, 2014). However, this is not always the case, and there are instances of the loss of force exceeding that of isometric stress (Brooks & Faulkner, 1991; Brown & Hasser, 1996). In the instances of the latter studies, very old animals (>24 months) are usually examined, representative of the final 20% of an animal's lifespan where significant muscle atrophy occurs (Brown & Hasser, 1996; Chan & Head, 2010). In our previous work, we showed no decline in soleus and EDL absolute force or absolute power between 10 weeks and 78 weeks of age (Hill *et al.*, 2018). Use of multiple ages in the present study allowed us to better observe the muscle ageing response, where we demonstrate that absolute force increases up to 52 weeks, prior to a decline in by 78 weeks, whilst isometric stress and power normalised to muscle mass continues to decline from peak maturity (Table 1), as with previous studies (Moran *et al.*, 2005; Chan & Head, 2010; Tallis *et al.*, 2014; Graber *et al.*, 2015; Hill *et al.*, 2018). Contradictory to human research showing a that the loss of

power is greater in magnitude than the loss of strength (Skelton *et al.*, 1994; Metter *et al.*, 1997; Krivickas *et al.*, 2001; Deschenes, 2004; Raj *et al.*, 2010), we report a significant decline in isometric stress that is faster than the decline in power normalised to muscle mass in early ageing for all muscles (10 weeks to 52 weeks) as with our previous work (Tallis *et al*., 2014). However, between 52 weeks and 78 weeks, this relationship is inversed and more closely reciprocates the aforementioned age-related changes in human muscle function. Thus, the current approach better captures the time-course of changes in absolute force, isometric stress, absolute power and normalised WL power. A rapid decline in absolute force and power to a magnitude that is similar to the more progressive loss of normalised force and power is observed by 78 weeks of age. This particular point has significant implications for muscle quality and overall muscle performance, as muscle mass is maintained but performed poorly compared to younger animals with a smaller muscle mass. As a consequence, larger muscles of poorer quality are maintained, increasing the metabolic demand for maintaining larger muscles, and could further exacerbate age-related muscle catabolism in older muscles should the metabolic energy requirements of the skeletal muscles not be met (Bottoni *et al.*, 2019).

Whilst our previous work reported age-related declines in maximal power output at a fixed CF (Tallis *et al.*, 2014; Hill *et al.*, 2018), the relationship between contractile velocity and WL power output had not been explored in ageing muscle. The present results show that there was a downward (i.e. reduced power output), but not leftward (i.e. shift to a slower CF) shift in either the absolute or normalised PO-CF curves in any muscles with increasing age. Graber *et al*. (2015) reported similar findings, in that absolute power of soleus and EDL exhibited a downward, but not leftward shift, in the force-power curve derived from isovelocity shortening contractions for male C57BL6 mice aged over 28 months. These findings are likely explained by the absence of an age-related fibre type change towards more oxidative characteristics nor significant fibre atrophy, supported by the slow/fast MHC isoform compositions being unaffected by age (Figure 8 A-I), no significant reduction in L_0 from 10 to 52 weeks (Table 1) and limited changes in activation and relaxation times (Figure 3 A-F). It is therefore likely that a change in the PO-CF relationship is only observed in very old age, where a substantial reduction in animal muscle mass (Brooks & Faulkner, 1988; Pagala *et al.*, 1998), a greater composition of oxidative fibres, and further shortening in muscle fibre length as is the trend for male EDL (Table 1), are likely to occur. Further work is required to

fully understand contractility responses of isolated muscles of very old (i.e. >78 weeks) outbred mice.

Ageing is more likely to affect skeletal muscle composed of predominantly fast-twitch muscle fibres as these have been found to be predisposed to a greater loss of contractile function due to a progressive alteration in fast fibre composition and the transition of fast fibres towards more oxidative fibre characteristics in humans (Klitgaard *et al.*, 1990; Deschenes, 2004) and type II fibre atrophy in rodents (Messa *et al.*, 2019). Previous work has shown that isometric stress of the EDL declines to a greater extent than the soleus with increasing age (Brooks & Faulkner, 1988; Brown & Hasser, 1996; Lynch *et al.*, 2001; Graber *et al.*, 2015), though this is not always the case (Moran *et al.*, 2005; Hill *et al.*, 2018). Our findings indicate that the severity of muscle ageing is sex and muscle-specific, where the loss of isometric muscle quality by 78 weeks of age is more greatly affected in male soleus than male EDL, whilst the inverse relationship is observed for females (Table 1). Moreover, the loss of muscle quality in the male soleus was greater than that of females where isometric stress declined significantly by as early as 30 weeks of age, with a similar rate of decline in muscle quality observed for sexes for the EDL and diaphragm. In further consideration of the sex-based differences in contractile performance, Chan & Head (2010) reported a significant reduction in EDL absolute force for 20-22-month-old 129/ReJ mouse EDL for females but not for males, whilst isometric stress declined equally between each sex irrespective of prevalent muscle atrophy. The present work differs to the findings of Chan & Head (2010), whereby soleus and EDL absolute force and stress is not different between males and females by 78 weeks of age, but the magnitude of the decline from the age which elicited maximal force production is greater in males than females. By 78 weeks of age soleus and EDL isometric stress was significantly lower in males than females, as is normalised power for all skeletal muscles examined. This difference between studies is likely a result of comparing animals of different ages and strains. Additionally, male EDL mass and muscle CSA continues to increase between 10 weeks and 52 weeks, without significant changes in absolute force and a continual decline in muscle quality; a phenomenon not observed in other muscles (Table 1). This may be due to a muscle-specific, sex-specific increase in intramuscular lipid accumulation otherwise not observed in humans (Delmonico *et al.*, 2009).

Unlike previous studies reporting no sex-based differences in transdiaphragmatic pressure of the diaphragm of old rodents (Greising *et al.*, 2015; Khurram *et al.*, 2018; Vang *et*

al., 2020) our data shows that power output normalised to muscle mass was greater in females than males by the oldest age (Figure 6 E&F; Table S1). The magnitude of the decline in diaphragm stress and normalised power with age was similar between each sex (Table 1) as with previous rodent studies (Greising *et al.*, 2015; Khurram *et al.*, 2018; Vang *et al.*, 2020). Contrary to the soleus and EDL, by 78 weeks of age diaphragm isometric stress plateaued, which is likely related to fibre hypertrophy as previously discussed (Messa *et al.*, 2019). These findings are contradictory to prior research using 23-month-old C57Bl/6 x 129 mice, where significant reductions in specific force were attributable to a 27% reduction in type IIx & IIb fibres (Greising *et al.*, 2015). Our data shows no change in slow or fast MHC isoforms for diaphragm by 78 weeks of age, which is a likely explanation for the disparity between results. The maintenance of force in CD-1 mice could be an adaptation in an attempt to preserve ventilatory function with increasing age, where an increased cost of breathing is observed in humans due to an age-related reduction in lung compliance and increased thoracic cavity stiffness (Sharma & Goodwin, 2006). However, diaphragm power output continued to decline by 78 weeks of age despite potential fibre hypertrophy (Messa *et al.*, 2019), with the requirement for dynamic power more important in respiration than isometric force production. This may imply that poorer diaphragmatic power is associated with greater negative work during a WL (i.e. greater eccentric activity during muscle relengthening; Figure S5 A-F) as a result of greater muscle stiffness, as observed in 79-week-old obese animals (Hill *et al.*, 2019).

Effects of Age and Sex on Fatigue Resistance

For the soleus, whilst age had an overall significant effect on fatigue resistance, this was only due to 3-week-old muscles being vastly more resistant to fatigue, and usually to a greater extent for females than males (Table S1), with no further differences observed between all other age groups of both sexes. The soleus is primarily composed of type I fibres, with the proportion and size of these fibres well maintained by 78 weeks of age (Messa *et al.*, 2019). 3-week-old muscles are also the most fatigue resistant for male EDL and diaphragm of both sexes, though no difference was observed for female EDL compared to 10 weeks and 78 weeks of age (Table 1). The change in contractile function for the soleus is likely due to a large increase in the size and number of type I fibres, where previous research in female Wistar rats found that the diameter and proportion of type I fibres for the soleus of post-weanling age

rats (21 days) were significantly greater than the type II fibre diameter and proportion (Cornachione *et al.*, 2011). The current findings are consistent with *in vivo* studies comparing fatigue resistance during high-intensity maximal contractions in children and adults, with children displaying markedly better fatigue resistance than adults, but at the expense of muscle strength (Kanehisa *et al.*, 1995; Zafeiridis *et al.*, 2005; Ratel *et al.*, 2006). This is primarily due to the greater proportion of type I fibres than type II fibres in children (Bell *et al.*, 1980; Oertel, 1988; Lexell *et al.*, 1992). Therefore, the age-related mechanical trade-off observed in 3-week-old muscles, where these muscles produce the least force and power but have the greatest fatigue resistance, is likely to be related the later development of fast-twitch fibres than slow-twitch fibres for post-weanling rodents.

Only male EDL and female diaphragm fatigue resistance was affected between 10 weeks and 78 weeks of age. For female diaphragm, the age-related decline in fatigue resistance was ongoing, whilst the pattern of fatigue for male EDL showed an initial decline in fatigue resistance between 10 weeks and 52 weeks, followed by a significant increase in fatigue resistance by 78 weeks, though a change in fibre type to a slower fibre composition is unlikely to account for this change (Figure 8 D, E & F). The ongoing decline in female diaphragm and male EDL fatigue resistance matched the progressive increase in relaxation times with age (Figure 3D & F), likely due to excitation-contraction uncoupling and impaired sarco(endo)plasmic reticulum-ATPase activity (Renganathan *et al.*, 1997; Tallis *et al.*, 2014). Irrespective of age, relaxation time increases with successive WL's during fatigue (Askew *et al.*, 1997). Therefore, the cumulative effect of age-related impairment in calcium resequestration during fatigue enhances negative work, where the muscle is still active during relengthening, and thus contributes to faster fatigue (Tallis *et al.*, 2014). Whilst the relative rate of fatigue between young and old muscles is similar for male diaphragm, female EDL and the soleus of both sexes, the maximal power output is lower at older ages, therefore it is likely that, coupled with an elevated body mass *in vivo*, the ability to sustain power is likely to be poorer *in vivo*, and even more so for female diaphragm and male EDL.

Mechanisms

Our data demonstrated no significant age-related changes in muscle mass for males, nor a change in slow or fast MHC isoforms for all skeletal muscles of both sexes (Figure 8 A-I) despite a loss of function in early age; indicative of dynapenia (Clark & Manini, 2008). In

humans, ageing is characterised by a 20-50% decline in type II fibre CSA in line with a loss of muscle strength and power, and before the loss of total muscle size, though type I fibres remain largely unchanged (Ballak *et al.*, 2014; Verdijk *et al.*, 2014). MHC atrophy may not be present when compared to older adults as 78 weeks old CD-1 mice may not yet be old enough to exhibit significant fibre atrophy, otherwise observed for our female CD-1 EDL mice (Messa *et al.,* 2019), with no other data in existence for comparison for this strain of mouse. For very old (i.e. >24 months) C57BL/6 male mice, no significant type I or type II fibre atrophy is observed for neither soleus (Brooks & Faulkner, 1994; Sheard & Anderson, 2012) or EDL (Sheard & Anderson, 2012). Age-related changes in F344*BN rats, however, report comparatively similar reductions in type II fibre CSA as humans (Ballak *et al.*, 2014) where this strain of rat can be tested up to the higher extremities of survivorship than the outbred CD-1 mouse.

Other mechanisms beyond muscle atrophy can contribute to the age-related loss of muscle quality, including age-related changes in hormones (Lowe *et al.*, 2010), muscle architecture (Kubo *et al.*, 2003), stiffening and increases in non-contractile tissues (McGregor *et al.*, 2014), excitation-contraction uncoupling (Renganathan *et al.*, 1997) and impaired actomyosin interactions (Lowe *et al.*, 2002). As a loss of muscle quality was observed before the loss of absolute function, with the latter accelerating in later life without prevalent fibre atrophy, the quantity of the non-contractile tissue, along with altered muscle architecture and efficiency of actin-myosin binding, are likely to be key contributors to the muscle ageing response which appears to affect male soleus to the greatest extent.

WL shapes indicated that male soleus & EDL, and female diaphragm, had greater negative work during muscle re-lengthening by loop 18 at 52 weeks and 78 weeks of age respectively (Figure S5 & Figure S6). Negative work during re-lengthening for female soleus & EDL was markedly lower, indicating lower resistance to stretch despite collagen not changing significantly in very old female humans (Inokuchi *et al.*, 1975). 78-week-old skeletal muscles also required length excursions across all CF's, as the larger sinusoidal length changes around L_0 as used in younger muscles significantly amplified negative work during muscle lengthening and relengthening, indicative of the age-related increase in muscle stiffness. Age-related increases in intramuscular adipose tissue (IMAT) and stiffening of the extracellular matrix due to increased collagen content have all been attributable to a decline in muscle quality (Kragstrup *et al.*, 2011; McGregor *et al.*, 2014) and increased passive stiffness (Lacraz *et al.*,

2015). Studies of the former have shown that intramuscular adipose tissue accumulation increases with age in humans (Kent-Braun *et al.*, 2000; Baumgartner, 2000; Frank-Wilson *et al.*, 2018) and up to 2.5 times more so than young adults (Kent-Braun *et al.*, 2000). One study measuring collagen content of human rectus abdominis, a non-weight bearing muscle, showed an increase up to the 50th decade for both sexes, but declines by the 80th for males but not females (Inokuchi *et al.*, 1975). Animal studies have consistently reported increased collagen content of rat soleus (Alnaqeeb *et al.*, 1984; Zimmerman *et al.*, 1993; Gosselin *et al.*, 1998) and EDL (Alnaqeeb *et al.*, 1984; Ramaswamy *et al.*, 2011), where collagen content of soleus increases to a greater extent than EDL (Alnaqeeb *et al.*, 1984) and the predominantly fast-twitch gastrocnemius (Zimmerman *et al.*, 1993). However, collagen content does not increase in mammalian diaphragm (Rodrigues *et al*., 1996) where a reduction in viscoelastic properties and an increase in collagen cross-linking are accountable for age-related muscle stiffness of this muscle.

Ageing causes a greater decline in fascicle length in older male skeletal muscles than females, consequently reducing the fascicle length and the number of sarcomeres over which muscle can produce force and power in males, (Kubo *et al.*, 2003). Whilst statistical significance was not observed, there was a trend towards a smaller optimal length for male soleus (Hedge's g = 0.78) and EDL (Hedge's g = 1.73), but not females, from 10 weeks to 78 weeks (Table 1). However, as muscle fibre length was not directly measured, this is only a speculative possibility. Another theory is that, at the cross-bridge level, ageing causes a 30% dissociation of myosin heads in the strong-binding state, leading to a 20% reduction in isometric force for male 32-37-month-old Fischer 344 x Brown Norway rats (Lowe *et al.*, 2002). During muscle shortening in a concentric contraction, the opportunities for actin-myosin binding sites to form are fewer than that during an isometric contraction, especially at faster contractile velocities (Lowe *et al.*, 2002). Should the level of myosin head dissociation be affected to the same extent during muscle shortening as during an isometric contraction, where force production is lower for the former compared to the latter, then poorer cross-bridge kinetics may explain the accelerated loss of power compared to force from 52 weeks to 78 weeks of age. Further work is required to fully elucidate the sex-based and muscle-specific mechanisms for poorer muscle quality in male murine skeletal muscles than females.

In Vivo Implications

As muscle size increases, the ability for muscles to overcome inertia decreases due to the proportional relationships between muscle force and CSA, and inertial loads (i.e. non-contractile tissues) and mass (i.e. body mass) (Ross & Wakeling, 2016). Therefore, age-related decreases in absolute power, increases in non-contractile mass and body mass, and maintenance of muscle CSA are all likely to further decrease the ability to overcome inertia. In our current work, we have identified a direct issue regarding an age-related loss of muscle quality at the isolated muscle level, which can be further exacerbated with respect to an elevated body mass. To better understand how the changes reported in this study may affect *in viv*o muscle function, power output was normalised to animal body mass (Figure 5 A-F), with absolute power output and power output normalised to muscle mass correlated with body mass (Figure S2 & S3) to determine whether larger animals had poorer power output and whether sex affected this relationship. Based on these results, older animals have poorer power output per unit of body mass, where males generated greater power output normalised to body mass than females in young age. By 78 weeks of age, however, female soleus generated greater power normalised to body mass than males, with no effect for sex for EDL. The magnitude of the decline in power normalised to body mass by 78 weeks of age was greater than the loss of absolute power and power output normalised to muscle mass (Table 1), which could signify significant *in vivo* complications for locomotory function. However, the regression analyses showed that isolated soleus and EDL from larger animals were able to generate greater absolute power output, but when normalised to muscle mass, no associations were found for all muscles.

We suggest that larger muscles producing greater absolute power would appear to be a requirement for overcoming larger body inertia, however, the heavier muscles of poorer quality contribute to an already elevated body mass, largely due to an age-related gain in visceral, ectopic, and subcutaneous fat mass (Ponti *et al.*, 2020), thus resulting in poorer power output per unit of body mass. Previous work has demonstrated that at the isolated muscle level, 9 weeks of a high-fat diet in 79-week-old female CD-1 mice resulted in a negative association between the maximal absolute power output of the soleus and animal body mass (Hill *et al.*, 2019). In humans, Visser *et al*. (2002) demonstrated that lower extremity performance is associated with greater fat infiltration into skeletal muscles for both men and

women. As such, significant age-related subcutaneous and intramuscular adiposity causes poorer associations between muscle function and body size and may help explain our current observations. Unfortunately, total fat mass and hindlimb mass was not measured in the present study to determine whether power output relative to this fat mass and limb mass was affected by age. It should be noted that by 78 weeks of age, absolute and normalised contractile performance is significantly reduced compared to all other ages, and more so for males compared to females for all muscles. The resultant effect is that older males have larger muscles that are contributing to an already elevated body mass but are of poorer quality. Coupled with an accelerated decline in absolute power output for the soleus and EDL, and poorer normalised power for the diaphragm, the effort of overcoming a greater limb mass and bodily inertia will be greater in males, and thus contributing to reducing overall locomotor performance. As a consequence, poorer ability to uptake and distribute oxygen, and to overcome bodily inertia, will likely significantly impact on the ability to complete activities of daily living.

Conclusion

The present study demonstrates that skeletal muscle ageing is complex and multifaceted, where the rate of loss of contractile mass and function is dependent on sex, the muscle examined (soleus, EDL and diaphragm), the mode of contraction (isometric twitch & tetani; acute & sustained WL power output) and normalisation of contractile performance to muscle mass and body mass. Studies to have focussed on specific elements of the muscle ageing response typically reported that male skeletal muscles age faster than females, the loss of power is more rapid than the loss of force and that fibres of a fast-twitch fibre composition are more greatly affected by age than predominantly slow-twitch skeletal muscles. The reported mechanical changes in these prior studies have been underpinned by age-related muscle atrophy and a significant alteration in muscle fibre composition. Our findings demonstrate a loss of muscle quality prior to the loss of absolute performance in early ageing, but an accelerated loss of absolute performance in later life, where the loss of power exceeds that of force in early ageing but is reversed in later life. These changes in muscle function are in lieu of an absence of significant skeletal muscle atrophy in most cases. As there was an absence of a shift in the PO-CF curves and an increase in fatigue resistance

with older age for male EDL, fibre type shifting may not be a key mechanism that elicits a reduction in contractile performance at the skeletal muscle level, as evidenced by no alteration in MHC isoforms. However, *in vivo* contractile function could be more limited in older males than females due to larger muscles of poorer quality contributing to an already elevated body inertia.

Conflict of Interest

The authors declare no conflict of interest.

Author Contributions

All authors approved the final version of the manuscript, agree to be accountable for all aspect of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved, and all persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

Author contributions: Conception, J. T. & C. H.; data acquisition, C. H. & F. S.; data analysis, C. H.; interpretation of data, C. H., R. S. J., V. M. C., F. S. & J. T.; drafting, C. H.; revisions; C. H., R. S. J., V. M. C., F. S. & J. T.

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Figure Titles

Figure 1 - Age-related changes in animal body mass (A), and muscle mass and muscle cross-sectional area (soleus, [SOL] B & C respectively; EDL, D & E respectively), for male (blue) and female (red) CD-1 mice aged 3-wks (n=8 for both sexes and muscles), 10-wks (n=10 for both sexes and muscles), 30-wks (n=8 for both muscles, male only), 52-wks (n=10 for female muscles; n=8 for male muscles) and 78-wks (n=8 for both sexes and muscles). Values presented as individual data along with mean ± S.D. Significant differences between each sex at a given age are indicated by common symbols; ** $p<0.01$, *** $p<0.001$.

Figure 2 - Age-related changes in soleus (SOL; A & B), EDL (C & D), and diaphragm (DIA; E) isometric tetanus force (A & C) and isometric tetanus stress (B, D & E) for male (blue) and female (red) CD-1 mice aged 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, male only), 52-wks (n=10 for all female muscles; n=8 for all male muscles) and 78-wks (n=8 for soleus & EDL of both sexes and female diaphragm, n=6 for male diaphragm). Values presented as individual data along with mean ± S.D. Significant differences between each sex at a given age are indicated by common symbols; * $p<0.05$.

Figure 3 - Age-related changes in soleus (SOL; A & B), EDL (C & D), and diaphragm (DIA; E & F) time to half-peak tetanus (THPT; A, C & E) and time from last stimulus to half-relaxation (LSHR; B, D & F) for male (blue) and female (red) CD-1 mice aged 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, male only), 52-wks (n=10 for female muscles; n=8 for male muscles) and 78-wks (n=8 for soleus & EDL of both sexes and female diaphragm, n=6 for male diaphragm). Values presented as individual data along with mean ± S.D. Significant differences between each sex at a given age are indicated by common symbols; * $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 4 - Age-related changes in soleus (SOL; A & B) and EDL (C & D) absolute power output across a range of cycle frequencies for male (blue) and female (red) CD-1 mice aged 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, male only), 52-wks (n=10 for female muscles; n=8 for male muscles) and 78-wks (n=8 all sexes and muscles). Values presented as mean ± S.D. Significant differences between each sex at a given age are indicated by common symbols; *** $p<0.001$.

Figure 5 - Age-related changes in soleus (SOL; A &B) and EDL (C & D) absolute power output normalised to body mass across a range of cycle frequencies for male (blue) and female (red) CD-1 mice aged 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, male only), 52-wks (n=10 for female muscles; n=8 for male muscles) and 78-wks (n=8 all sexes and muscles). Values presented as mean ± S.D. Significant differences between each sex at a given age are indicated by common symbols; *$p<0.05$, *** $p<0.001$.

Figure 6 - Age-related changes in soleus (SOL; A & B), EDL (C & D) and diaphragm (E & F) power output normalised to muscle mass across a range of cycle frequencies for male (blue) and female (red) CD-1 mice aged 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, male only), 52-wks (n=10 for female muscles; n=8 for male muscles) and 78-wks (n=8 for soleus & EDL of both sexes and female diaphragm, n=6 for male diaphragm). Values presented as mean ± S.D. Significant differences between each sex at a given age are indicated by common symbols; * $p<0.05$, *** $p<0.001$.

Figure 7 – The effect of age and sex on the ability to sustain power for male (blue) and female (red) soleus (SOL; A & B), EDL (C & D), and diaphragm (DIA; E & F) at 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, males only) 52-wks (n=10 for female muscles, n=8 for male muscles) and 78-wks (n=8 for soleus & EDL of both sexes and female diaphragm, n=6 for male diaphragm). Values presented as mean ± S.D. Significant differences between each sex at a given age are indicated by common symbols; ** $p<0.01$.

Figure 8 – Biochemical analyses of slow MHC/α-tubulin (A, D & G), fast MHC/α-tubulin (B, E & H), and slow/fast MHC (C, F & I) for male (blue) and female (red) soleus (SOL; A, B & C), EDL (D, E & F) and diaphragm (DIA; G, H & I) aged 10 weeks and 78 weeks. Values presented as individual data along with mean ± S.D. N=6 per muscle, per sex, per age. MHC, myosin heavy chain.

Table Legends

Table 1 – Percentage differences in animal morphology, isometric properties and WL power output from the age at which the maximal measurement for each variable occurred. Values presented as mean. A * denotes significant ($P<0.05$) differences from the "Max" value. For time to half-peak tetanus (THPT) and last stimulus to half relaxation (LSHR), the "Max" value represents the age at which muscle activation and relaxation was fastest (i.e. the smallest value).

L_0, optimal muscle length; CSA, muscle cross-sectional area; Norm., normalised; BM, body mass; MM, muscle mass.

Supplementary Figure Titles

Figure S1 – Time-course of recovery of power output relative to the pre-fatigue maximal power output every 10 minutes following the fatigue protocol for male (blue) and female (red) soleus (SOL; A & B), EDL, (C & D) and diaphragm (DIA; E & F) aged 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, males only) 52-wks (n=10 for female muscles, n=8 for male muscles) and 78-wks (n=8 for soleus & EDL of both sexes and female diaphragm, n=6 for male diaphragm). Values presented as mean ± S.D.

Figure S2 - Regression analyses of whole animal body mass compared with absolute power output at a cycle frequency of 5Hz for male (blue) and female (red) soleus (SOL; A & B) and 10Hz for EDL (C & D) aged 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, male only), 52-wks (n=10 for female muscles; n=8 for male muscles) and 78-wks (n=8 all sexes and muscles). Data are fitted with a first-order polynomial using least squares regressions and 95% confidence limits for these lines.

Figure S3 - Regression analyses of whole animal body mass compared with power output normalised to muscle mass for males (blue) and females (red) at a cycle frequency of 5Hz for soleus (SOL; A & B), 10Hz for EDL (C & D) and 7Hz for diaphragm (DIA; E & F) aged 3-wks (n=8 for all sexes and muscles), 10-wks (n=10 for all sexes and muscles), 30-wks (n=8 for all muscles, male only), 52-wks (n=10 for female muscles; n=8 for male muscles) and 78-wks (n=8 for soleus & EDL of both sexes and female diaphragm, n=6 for male diaphragm).

Figure S4 – Age-related changes in work loop shapes during the fatigue protocol at 5Hz cycle frequency for male and female soleus aged 3-wks (A & B), 10-wks (C & D), 52-wks (E & F) and 78-wks (G & H). Work loops 2 (0.4 secs), 10 (2.0 secs) and 18 (3.6 secs) of the fatigue protocol are shown for each group. Work loops are interpreted in the anti-clockwise direction from 0% of L_0. Work loop shapes did not alter for 3-wk soleus where fatigue resistance was best, though further ageing did not cause a greater eccentric activity during relengthening and as such fatigue resistance was not significantly altered.

Figure S5 – Age-related changes in work loop shapes during the fatigue protocol at 10Hz cycle frequency for male and female EDL aged 3-wks (A & B), 10-wks (C & D), 52-wks (E & F) and 78-wks (G & H). Work loops 2 (0.2 secs), 10 (1.0 secs) and 18 (1.8 secs) of the fatigue protocol are shown for each group. Work loops are interpreted in the anti-clockwise direction from 0% of L_0. Eccentric muscle activity during muscle relengthening was increased from 3-wk to 52-week-old EDL, where fatigue resistance was poorest. By 78-wks, there was less eccentric work during relengthening, which was associated with improved fatigue resistance.

Figure S6 – Age-related changes in work loop shapes during the fatigue protocol at 7Hz cycle frequency for male and female diaphragm aged 3-wks (A & B), 10-wks (C & D), 52-wks (E & F) and 78-wks (G & H). Work loops 2 (0.28 secs), 10 (1.4 secs) and 18 (2.52 secs) of the fatigue protocol are shown for each group. Work loops are interpreted in the anti-clockwise direction from 0% of L_0. Female diaphragm produced substantially more eccentric work during relengthening by 78-weeks of age, where fatigue resistance was poorest.

Supplementary Table Legends

Table S1: A list of all the computed p-values for male and female CD-1 mouse body mass, muscle morphology, isometrics and the work loop power output-cycle frequency (PO-CF) relationship for soleus, EDL and diaphragm, with sex set as the main effect. All variables tested using ANOVA's (two-way ANOVA - morphology & isometrics; three-way ANOVA – work loop PO-CF) with Tukey's post-hoc analysis used to compare the dependent variables of each sex at a specific age. Values reported to 2 decimal places/significant figures.

SOL, soleus; EDL; extensor digitorum longus; DIA, diaphragm; L_0, optimal muscle length CSA, cross-sectional area; THPT, time to half-peak tetanus; LSHR, last stimulus to half-relaxation.

[a] Mean value for the given variable is significantly greater for males than females.

[b] Mean value for the given variable is significantly greater for females than males.

Table S2: A list of all the computed p-values for female CD-1 mouse body mass and muscle morphology, isometrics and work loop power output-cycle frequency (PO-CF) relationship for soleus, EDL and diaphragm with age set as the main effect. All variables tested using ANOVA's (two-way ANOVA - morphology & isometrics; three-way ANOVA - PO-CF) with Tukey's post-hoc analysis used to compare the dependent variables of between each age for females.

SOL, soleus; EDL; extensor digitorum longus; DIA, diaphragm; L_0, optimal muscle length; CSA, cross-sectional area; THPT, time to half-peak tetanus; LSHR, last stimulus to half-relaxation; PO.BM, power output relative to body mass.

Table S3: A list of all the computed p-values for male CD-1 mouse body mass and muscle morphology, isometrics and work loop power output-cycle frequency (PO-CF) relationship for soleus, EDL and diaphragm with age set as the main effect. All variables tested using ANOVA's (two-way ANOVA - morphology & isometrics; three-way ANOVA - PO-CF) with Tukey's post-hoc analysis used to compare the dependent variables of between each age for males. Values reported to 2 decimal places/significant figures.

SOL, soleus; EDL; extensor digitorum longus; DIA, diaphragm; L_0, optimal muscle length; CSA, cross-sectional area; THPT, time to half-peak tetanus; LSHR, last stimulus to half-relaxation; PO.BM, power output relative to body mass.

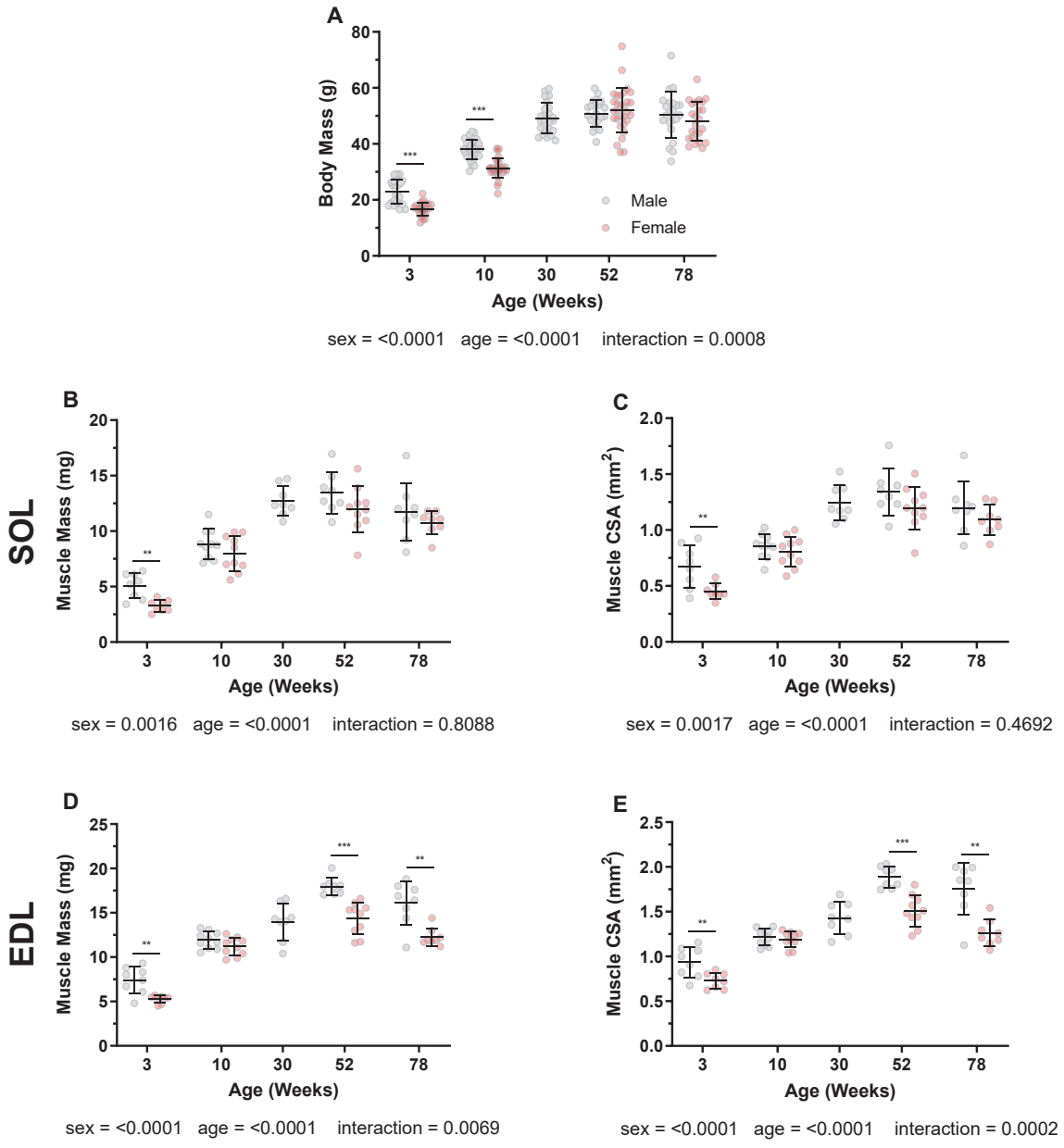

A
Body Mass (g)
Male
Female
Age (Weeks)
sex = <0.0001 age = <0.0001 interaction = 0.0008
B
SOL
Muscle Mass (mg)
Age (Weeks)
sex = 0.0016 age = <0.0001 interaction = 0.8088
C
Muscle CSA (mm2)
Age (Weeks)
sex = 0.0017 age = <0.0001 interaction = 0.4692
D
EDL
Muscle Mass (mg)
Age (Weeks)
sex = <0.0001 age = <0.0001 interaction = 0.0069
E
Muscle CSA (mm2)
Age (Weeks)
sex = <0.0001 age = <0.0001 interaction = 0.0002

Figure 1

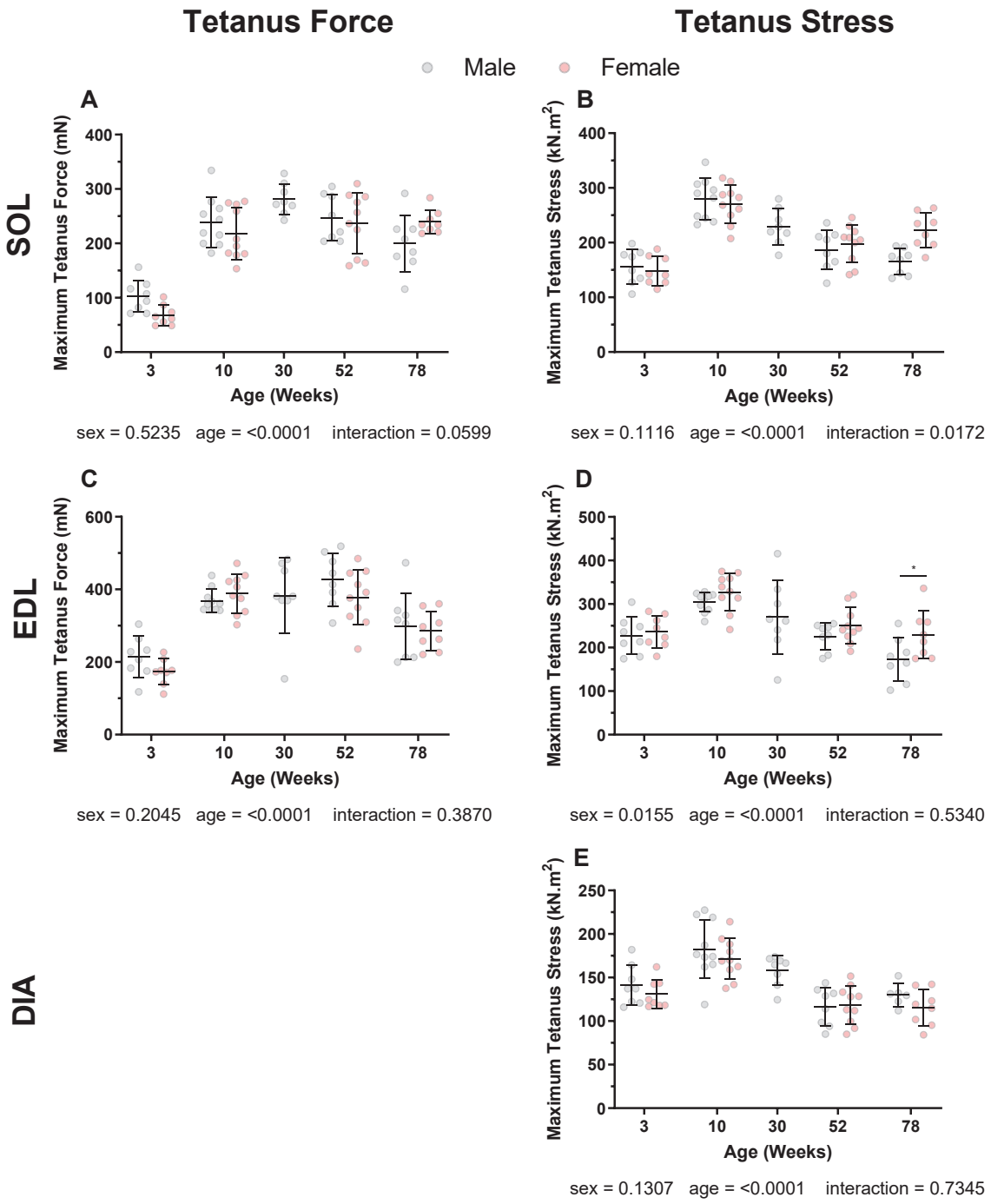

Tetanus Force
Tetanus Stress
Male
Female
SOL
EDL
DIA
A
B
C
D
E
Maximum Tetanus Force (mN)
Maximum Tetanus Stress (kN.m^2)
Age (Weeks)
3
10
30
52
78
sex = 0.5235 age = <0.0001 interaction = 0.0599
sex = 0.1116 age = <0.0001 interaction = 0.0172
sex = 0.2045 age = <0.0001 interaction = 0.3870
sex = 0.0155 age = <0.0001 interaction = 0.5340
sex = 0.1307 age = <0.0001 interaction = 0.7345

Figure 2

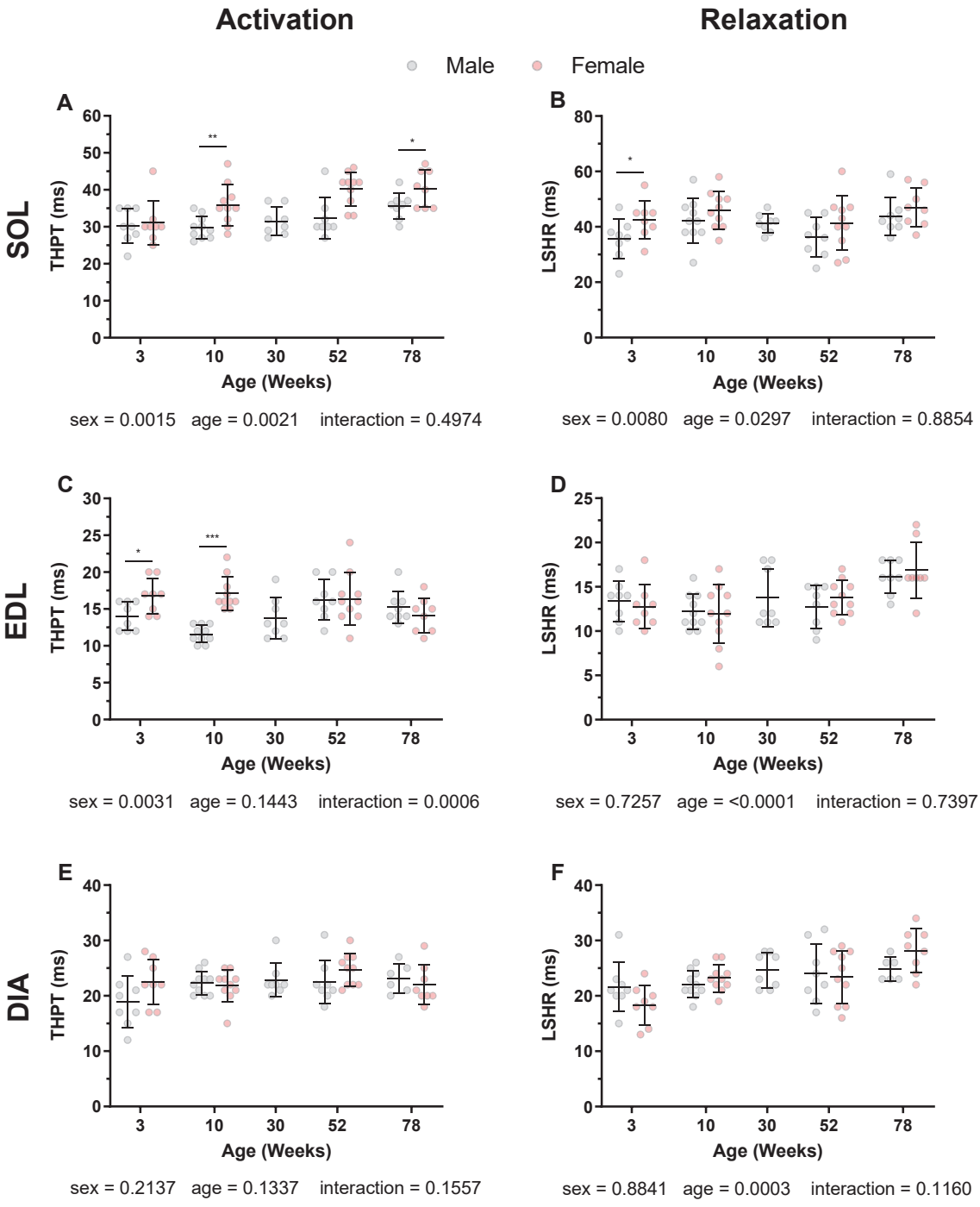

Activation
Relaxation
Male
Female
A
B
C
D
E
F
SOL
EDL
DIA
THPT (ms)
LSHR (ms)
Age (Weeks)
3
10
30
52
78
sex = 0.0015 age = 0.0021 interaction = 0.4974
sex = 0.0080 age = 0.0297 interaction = 0.8854
sex = 0.0031 age = 0.1443 interaction = 0.0006
sex = 0.7257 age = <0.0001 interaction = 0.7397
sex = 0.2137 age = 0.1337 interaction = 0.1557
sex = 0.8841 age = 0.0003 interaction = 0.1160

Figure 3

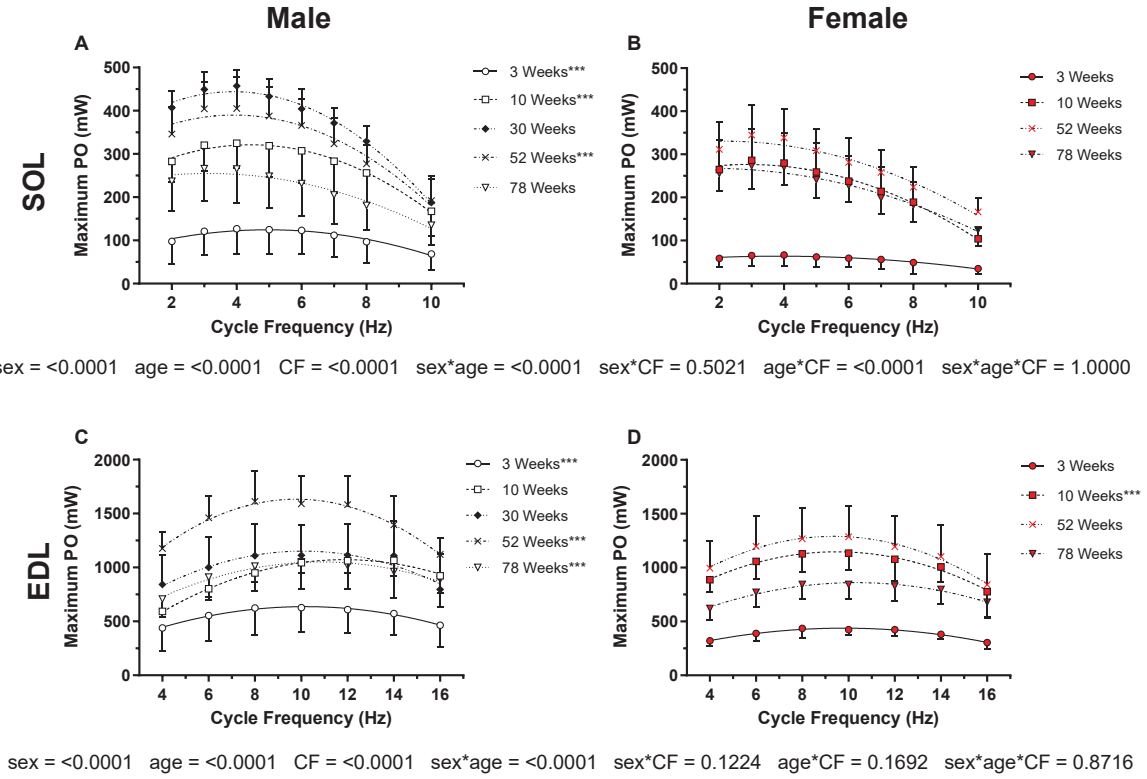

Male
Female
A
B
SOL
Maximum PO (mW)
Cycle Frequency (Hz)
3 Weeks***
10 Weeks***
30 Weeks
52 Weeks***
78 Weeks
3 Weeks
10 Weeks
52 Weeks
78 Weeks
sex = <0.0001 age = <0.0001 CF = <0.0001 sex*age = <0.0001 sex*CF = 0.5021 age*CF = <0.0001 sex*age*CF = 1.0000
C
D
EDL
3 Weeks***
10 Weeks
30 Weeks
52 Weeks***
78 Weeks***
3 Weeks
10 Weeks***
52 Weeks
78 Weeks
sex = <0.0001 age = <0.0001 CF = <0.0001 sex*age = <0.0001 sex*CF = 0.1224 age*CF = 0.1692 sex*age*CF = 0.8716

Figure 4

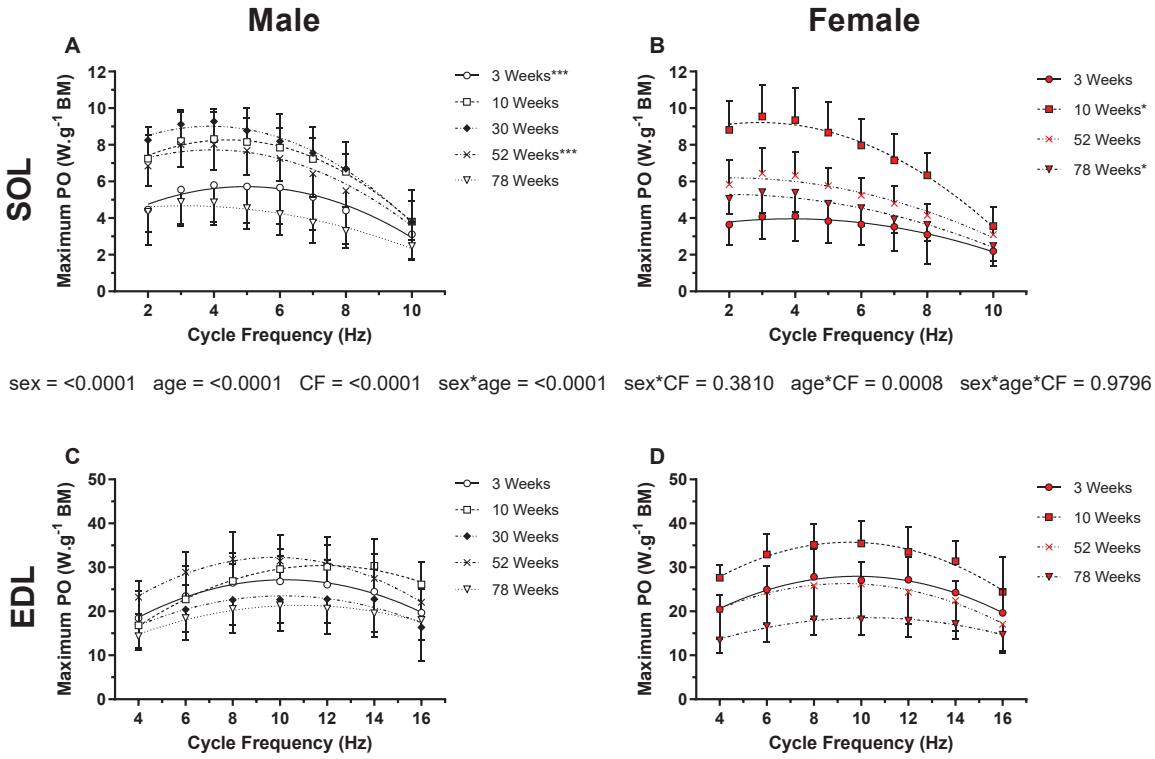
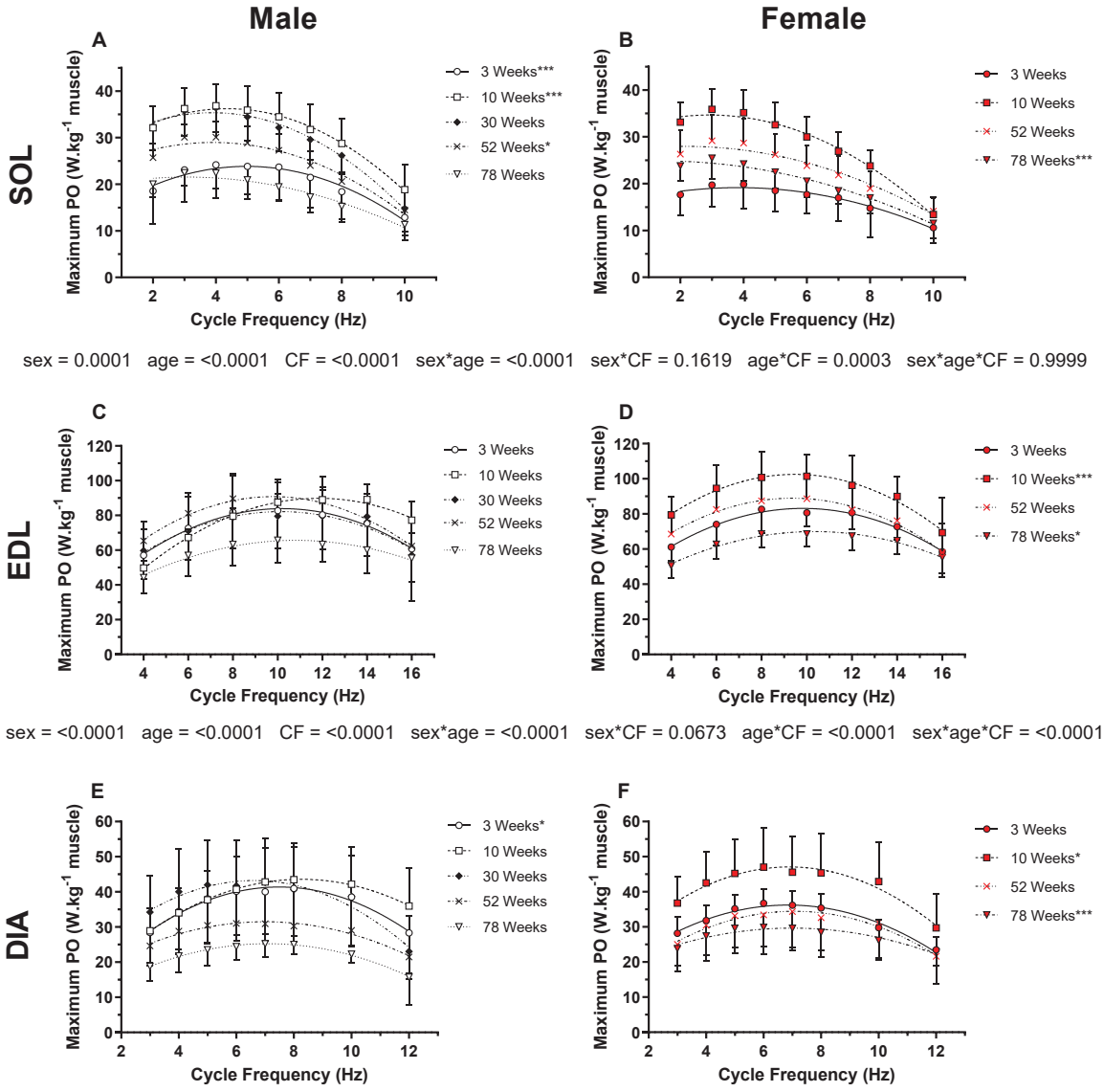

Male
Female
A
B
SOL
Maximum PO (W.g-1 BM)
Cycle Frequency (Hz)
3 Weeks***
10 Weeks
30 Weeks
52 Weeks***
78 Weeks
3 Weeks
10 Weeks*
52 Weeks
78 Weeks*
sex = <0.0001 age = <0.0001 CF = <0.0001 sex*age = <0.0001 sex*CF = 0.3810 age*CF = 0.0008 sex*age*CF = 0.9796
C
D
EDL
Maximum PO (W.g-1 BM)
Cycle Frequency (Hz)
3 Weeks
10 Weeks
30 Weeks
52 Weeks
78 Weeks
sex = 0.5414 age = <0.0001 CF = <0.0001 sex*age = <0.0001 sex*CF = 0.1197 age*CF = 0.7805 sex*age*CF = 0.7908

Figure 5



sex = 0.0001 age = <0.0001 CF = <0.0001 sex*age = <0.0001 sex*CF = 0.1619 age*CF = 0.0003 sex*age*CF = 0.9999

sex = <0.0001 age = <0.0001 CF = <0.0001 sex*age = <0.0001 sex*CF = 0.0673 age*CF = <0.0001 sex*age*CF = <0.0001

sex = 0.0132 age = <0.0001 CF = <0.0001 sex*age = 0.0001 sex*CF = 0.6485 age*CF = 0.9378 sex*age*CF = 0.9907

Figure 6

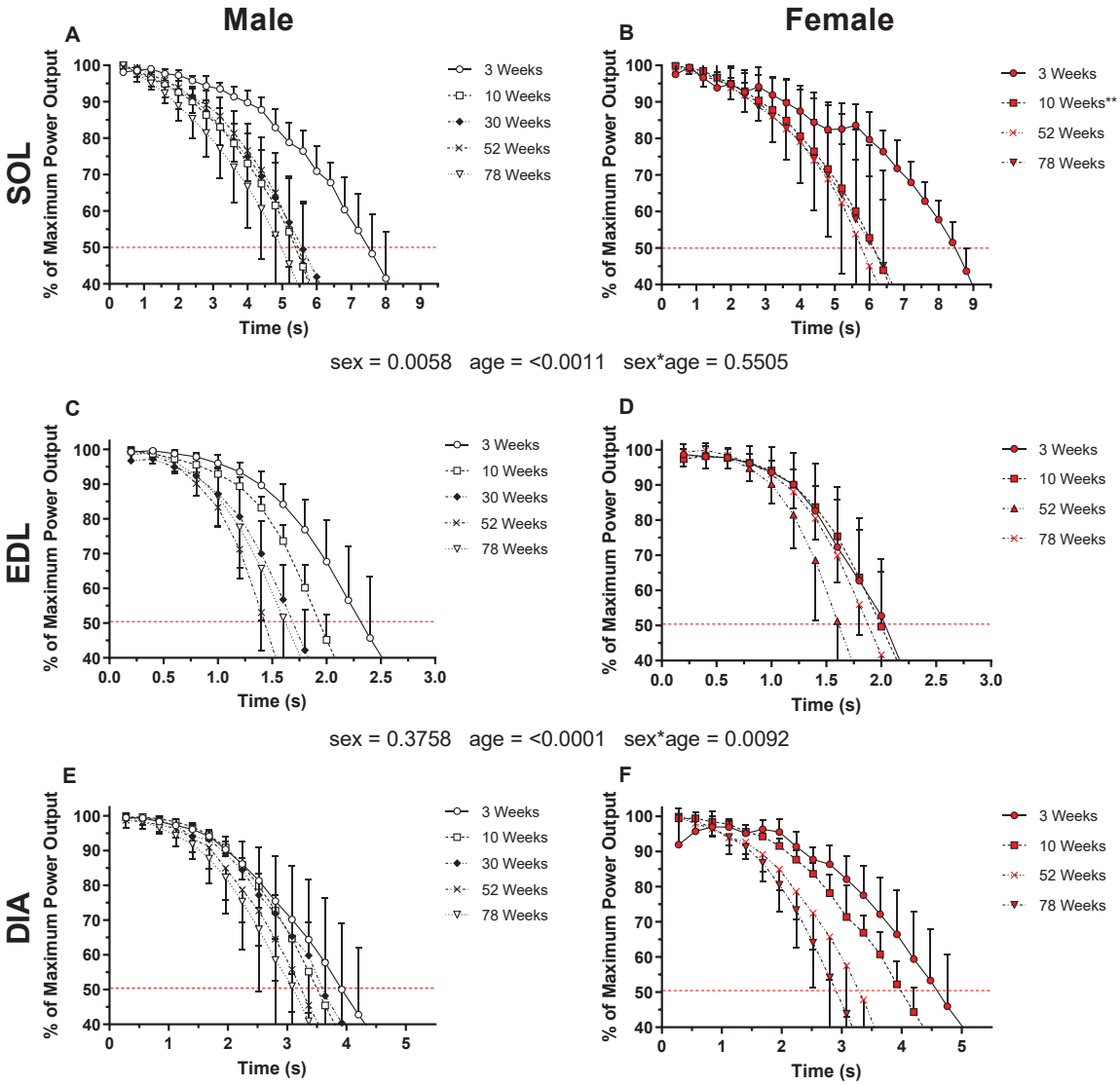

Male
Female
A
B
SOL
% of Maximum Power Output
Time (s)
3 Weeks
10 Weeks
30 Weeks
52 Weeks
78 Weeks
10 Weeks**
sex = 0.0058 age = <0.0011 sex*age = 0.5505
C
D
EDL
sex = 0.3758 age = <0.0001 sex*age = 0.0092
E
F
DIA
sex = 0.2057 age = <0.0001 sex*age = 0.0266

Figure 7

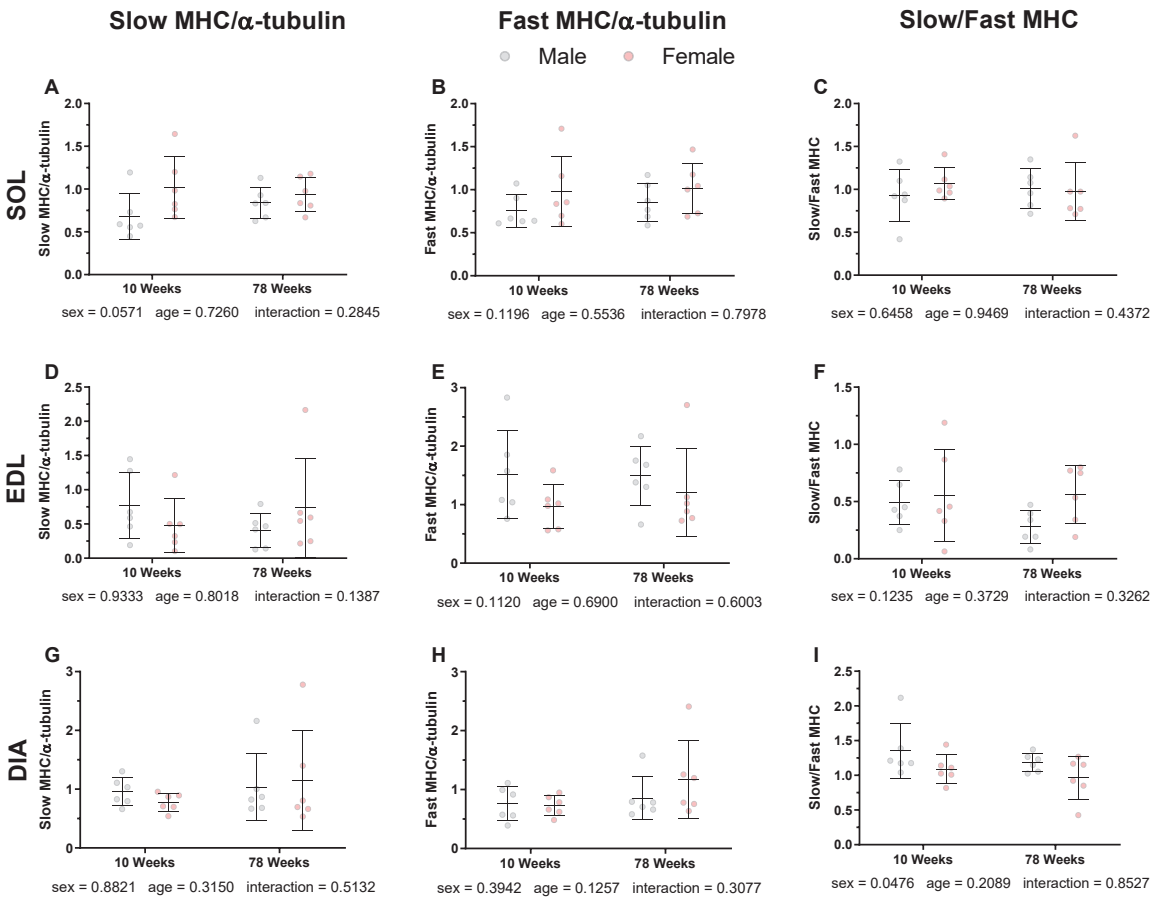

Slow MHC/α-tubulin
Fast MHC/α-tubulin
Slow/Fast MHC
Male
Female
A
B
C
D
E
F
G
H
I
SOL
EDL
DIA
Slow MHC/α-tubulin
Fast MHC/α-tubulin
Slow/Fast MHC
10 Weeks
78 Weeks
sex = 0.0571 age = 0.7260 interaction = 0.2845
sex = 0.1196 age = 0.5536 interaction = 0.7978
sex = 0.6458 age = 0.9469 interaction = 0.4372
sex = 0.9333 age = 0.8018 interaction = 0.1387
sex = 0.1120 age = 0.6900 interaction = 0.6003
sex = 0.1235 age = 0.3729 interaction = 0.3262
sex = 0.8821 age = 0.3150 interaction = 0.5132
sex = 0.3942 age = 0.1257 interaction = 0.3077
sex = 0.0476 age = 0.2089 interaction = 0.8527

Figure 8

	Age								
	Male					Female			
	3 weeks	10 weeks	30 weeks	52 weeks	78 weeks	3 weeks	10 weeks	52 weeks	78 weeks
					Combined				
Mass (g)	-56%*	-25%*	-3%	Max	-1%	-68%*	-40%*	Max	-8%
					Soleus				
samples	8	10	8	8	8	8	10	10	8
Mass (mg)	-62%*	-34%*	-6%	Max	-13%	-73%*	-33%*	Max	-10%
)	-25%*	0%	Max	-2%	-4%	-27%	-2%	Max	-1%
CSA (m^2)	-50%*	-36%*	-7%	Max	-11%	-62%*	-33%*	Max	-9%
Force (mN)	-65%*	-10%	Max	-7%	-31%*	-58%*	-17%	Max	-11%
Stress ($kN.m^2$)	-49%*	Max	-23%*	-34%*	-46%*	-12%	Max	-19%	-21%
s Force (mN)	-63%*	-15%	Max	-12%	-29%*	-72%*	-9%	-1%	Max
s Stress ($kN.m^2$)	-44%*	Max	-18%*	-33%*	-41%*	-45%*	Max	-27%*	-18%*
ms)	2%	Max	6%	13%	20%*	Max	15%	26%*	30%*
ms)	Max	18%	16%	2%	23%	3%	11%	Max	14%
te Power (mW)	-71%*	-26%*	Max	-10%*	-41%*	-80%*	-21%*	Max	-23%*
Power ($W.g^{-1}$ BM)	-35%*	-7%	Max	-13%*	-47%*	-54%*	Max	-31%*	-43%*
Power ($W.kg^{-1}$ MM)	-34%*	Max	-7%	-20%*	-41%*	-39%*	Max	-15%*	-27%*
o 50% Fatigue (s)	Max	-27%*	-25%*	-26%*	-33%*	Max	-21%*	-24%*	-21%*
					EDL				
samples	8	10	8	8	8	8	10	10	8
Mass (mg)	-59%*	-34%*	-22%*	Max	-10%	-63%*	-22%*	Max	-15%*
)	-19%*	Max	-1%	-3%	-6%	-26%*	-4%	-2%	Max
CSA (m^2)	-50%*	-36%*	-24%*	Max	-7%	-52%*	-21%*	Max	-16%*
Force (mN)	-52%*	-11%	-10%	Max	-22%	-67%*	-2%	Max	-18%

Stress (kN.m^2)	-33%*	Max	-13%	-28%*	-38%*		-44%*	Max	-20%*	-20%*
ıs Force (mN)	-50%*	-14%	-10%	Max	-30%*		-56%*	Max	-3%	-27%*
ıs Stress (kN.m^2)	-25%*	Max	-11%	-26%*	-42%*		-28%*	Max	-23%*	-30%*
ms)	21%	Max	19%	40%*	31%*		19%	21%	16%	Max
ms)	10%	Max	13%	5%	32%*		7%	Max	16%	42%*
ıte Power (mW)	-61%*	-35%*	-29%*	Max	-34%*		-66%*	-10%	Max	-31%*
Power (W.g^{-1} BM)	-15%	-7%	-25%	Max	-31%		-22%	Max	-27%	-47%
Power (W.kg^{-1} MM)	-7%	-2%	-8%	Max	-26%*		-19%*	Max	-14%*	-30%*
o 50% Fatigue (s)	Max	-18%*	-28%*	-37%*	-31%*		Max	-1%	-19%*	-6%
					Diaphragm					
samples	8	10	8	8	6		8	10	10	8
Stress (kN.m^2)	-20%	Max	-4%	-24%*	-8%		-19%	Max	-31%*	-23%*
ıs Stress (kN.m^2)	-23%*	Max	-13%	-36%*	-29%*		-24%*	Max	-31%*	-33%*
ms)	Max	18%	21%	19%	23%		3%	Max	13%	1%
ms)	Max	2%	14%	11%	15%		Max	26%*	27%*	53%*
Power (W.kg^{-1} MM)	-5%	Max	-1%	-25%*	-41%*		-23%*	Max	-27%*	-34%*
o 50% Fatigue (s)	Max	-10%	-8%	-17%	-13%		Max	-16%*	-28%*	-36%*

Table 1 – Mean percentage differences in animal morphology, isometric properties and WL power output from the age at which the maximal measurement for each variable occurred. Values presented as mean. A * denotes significant ($P<0.05$) differences from the "Max" value. For time to half-peak tetanus (THPT) and last stimulus to half relaxation (LSHR), the "Max" value represents the age at which muscle activation and relaxation was fastest (i.e. the smallest value).

L_0, optimal muscle length; CSA, muscle cross-sectional area; Norm., normalised; BM, body mass; MM, muscle mass.

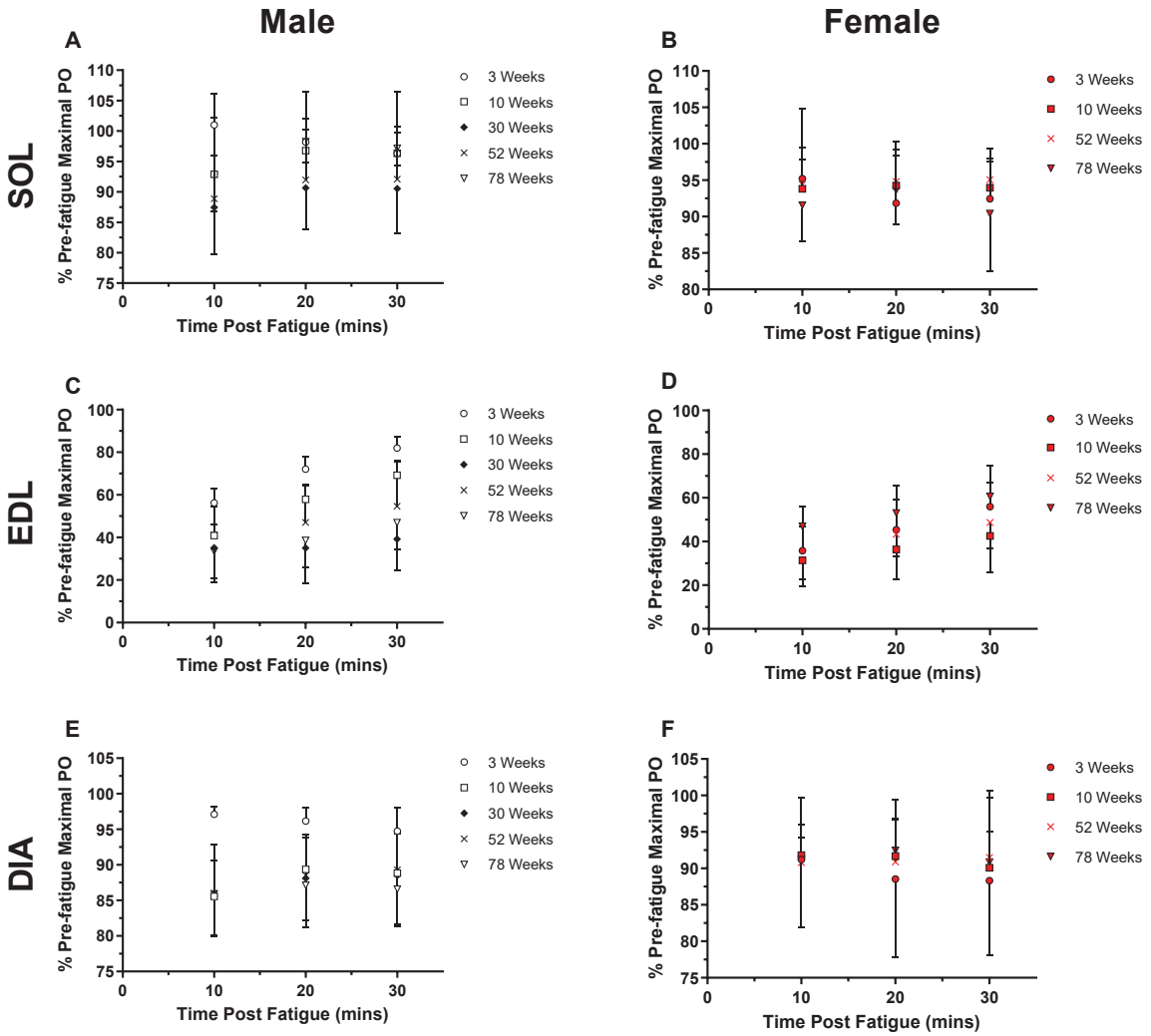

Male
Female
A
B
C
D
E
F
SOL
EDL
DIA
% Pre-fatigue Maximal PO
Time Post Fatigue (mins)
3 Weeks
10 Weeks
30 Weeks
52 Weeks
78 Weeks

Figure S1

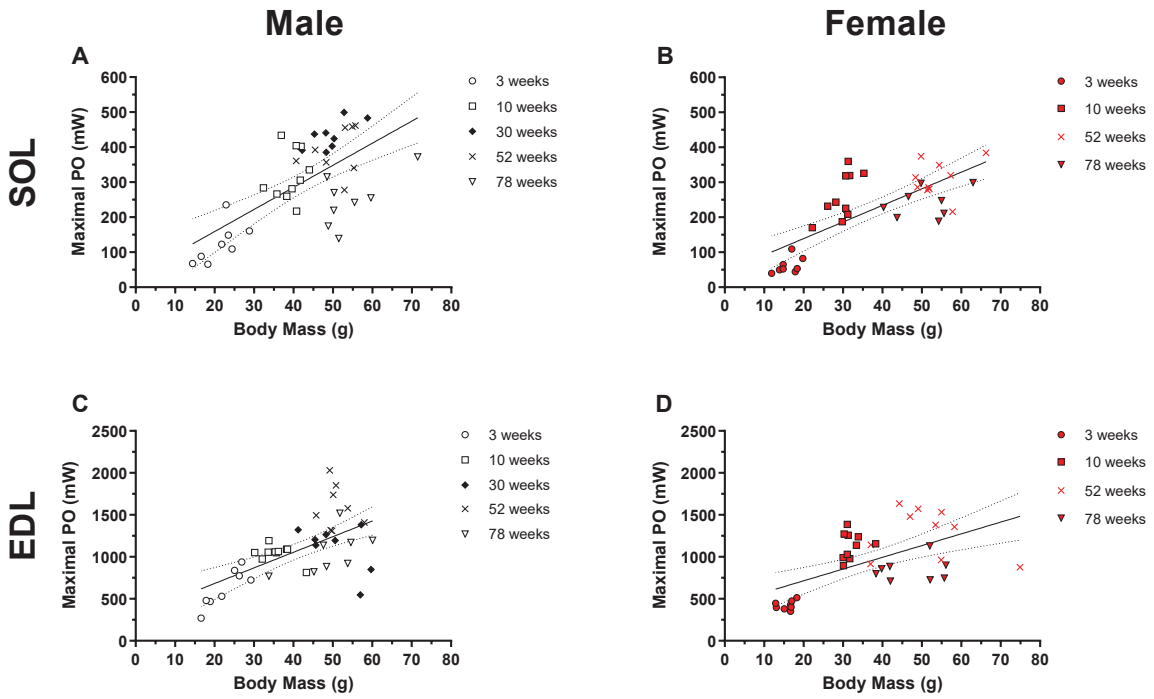

Male
Female
A
B
C
D
SOL
EDL
Maximal PO (mW)
Body Mass (g)
3 weeks
10 weeks
30 weeks
52 weeks
78 weeks

Figure S2

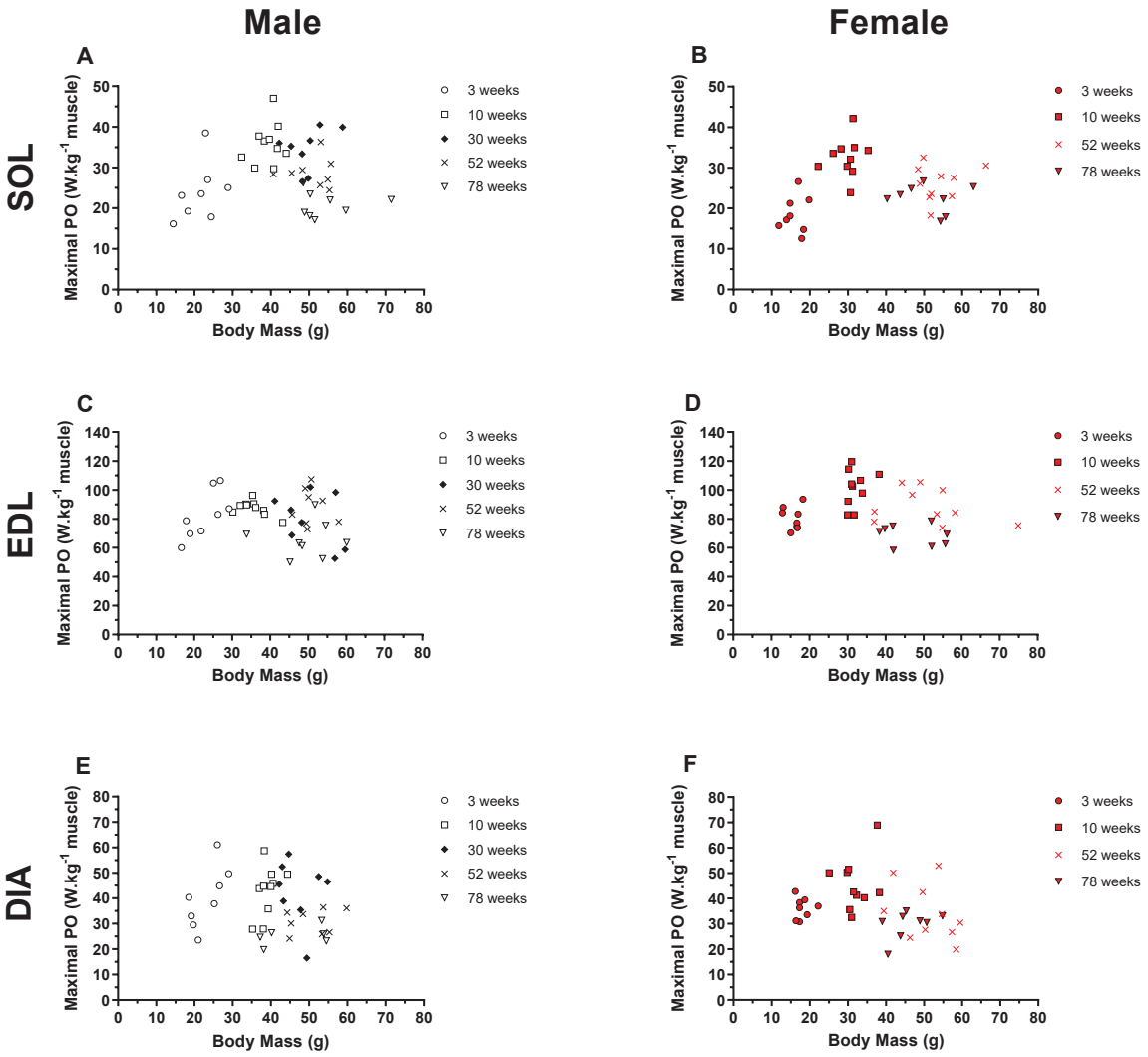

Male
Female
A
B
C
D
E
F
SOL
EDL
DIA
Maximal PO (W.kg-1 muscle)
Body Mass (g)
3 weeks
10 weeks
30 weeks
52 weeks
78 weeks

Figure S3

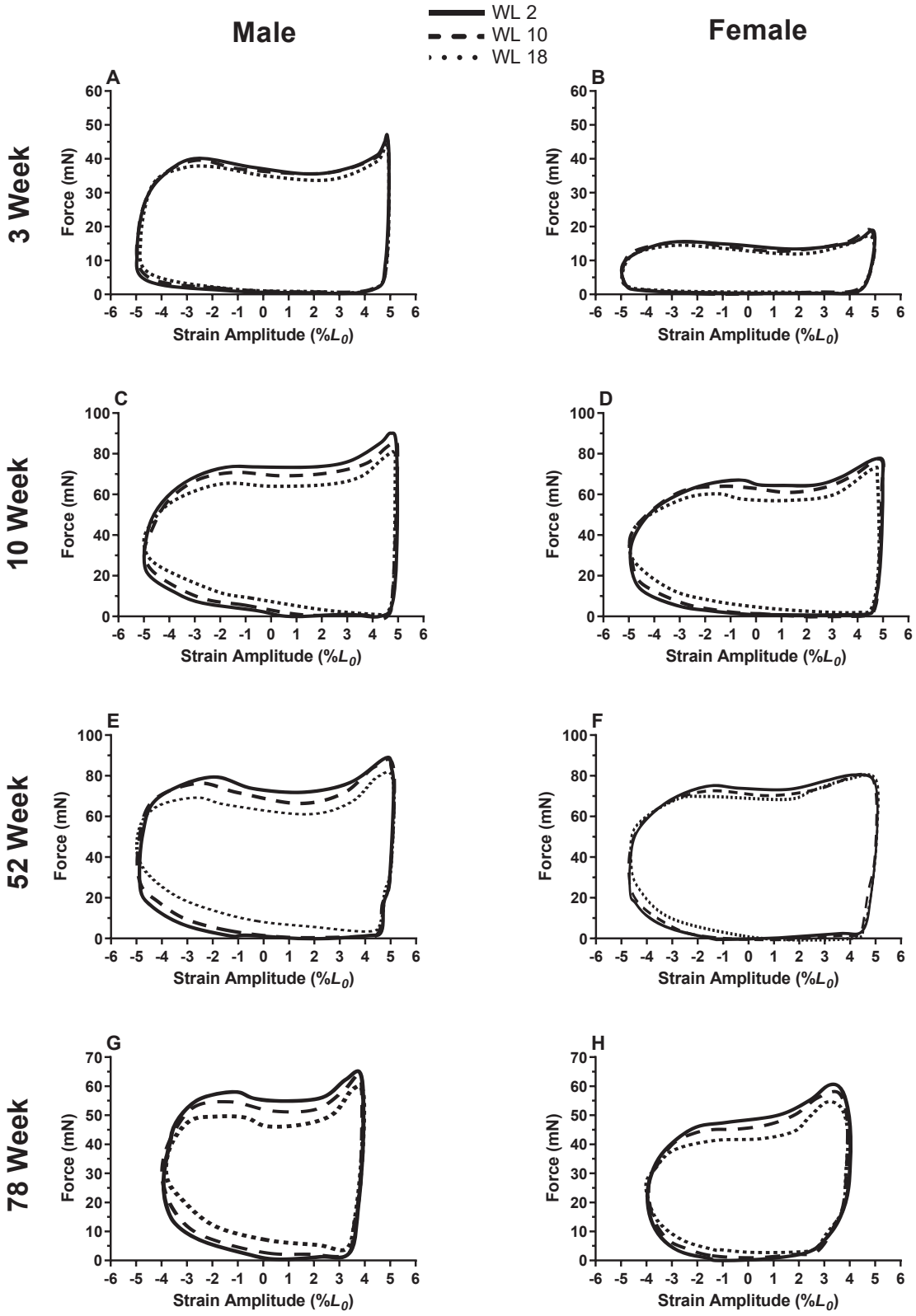

WL 2
WL 10
WL 18
Male
Female
3 Week
10 Week
52 Week
78 Week
A
B
C
D
E
F
G
H
Force (mN)
Strain Amplitude (%L_0)

Figure S4

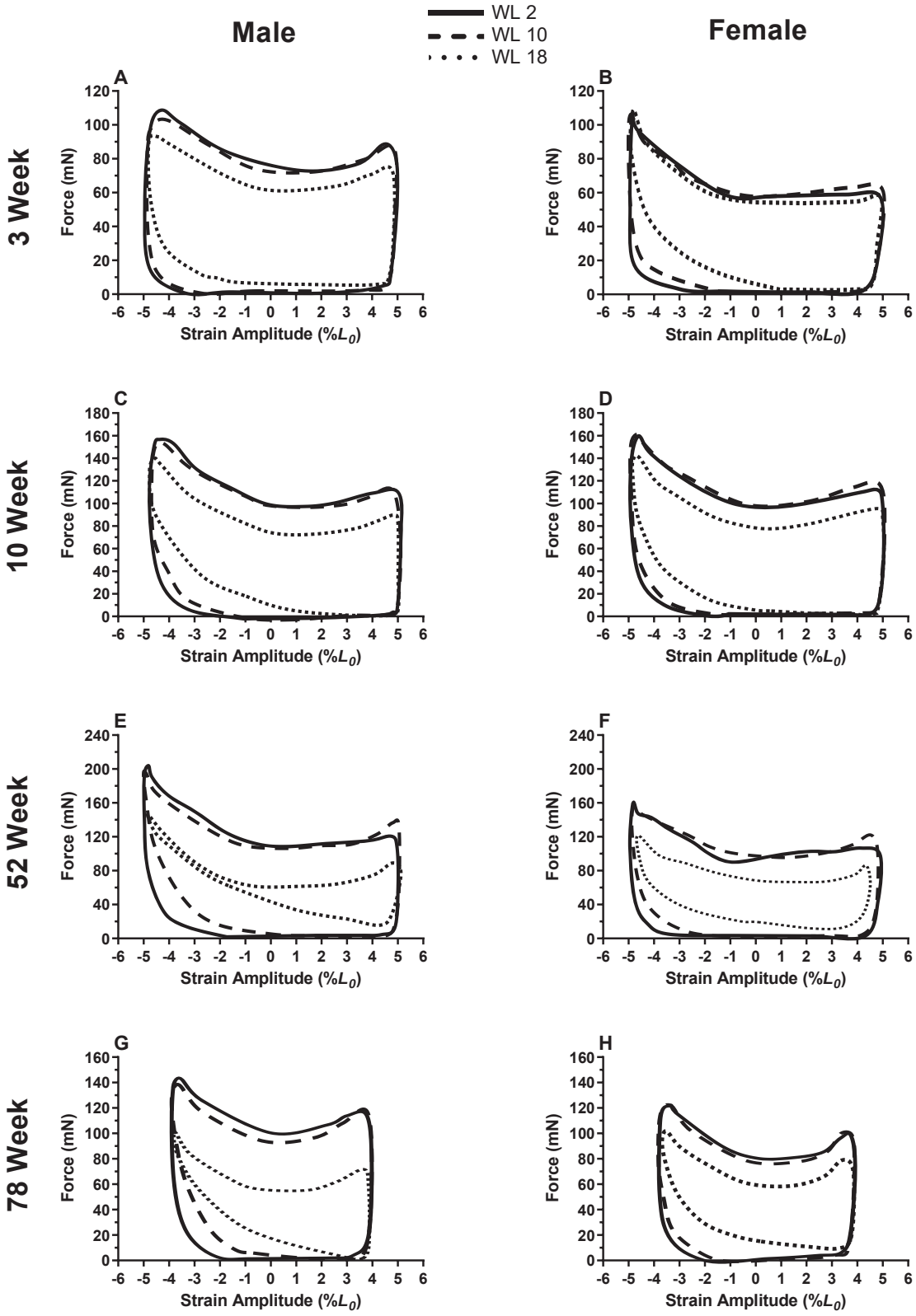

WL 2
WL 10
WL 18
Male
Female
3 Week
10 Week
52 Week
78 Week
A
B
C
D
E
F
G
H
Force (mN)
Strain Amplitude (%L_0)

Figure S5

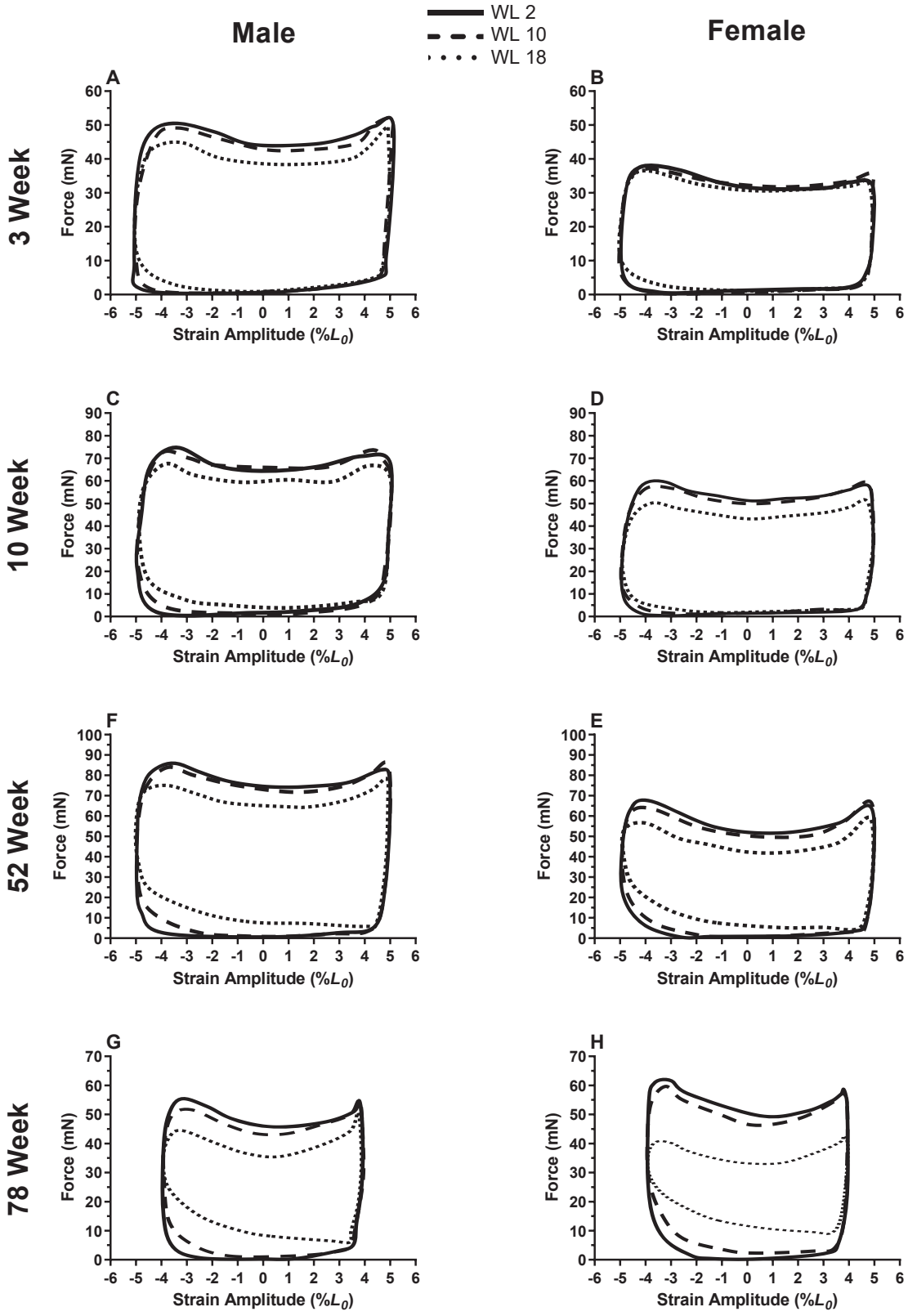

WL 2
WL 10
WL 18
Male
Female
3 Week
10 Week
52 Week
78 Week
A
B
C
D
E
F
G
H
Force (mN)
Strain Amplitude (%L_0)

Figure S6

	P-Values					*Effect Size*			
Variable	**Main Effect (Sex)**	**3 Weeks**	**10 Weeks**	**52 Weeks**	**78 Weeks**	**3 Weeks**	**10 Weeks**	**52 Weeks**	**78 Weeks**
				Morphology					
Body Mass	<0.0001	<0.0001^{M}	<0.0001^{M}	0.5085	0.3091	1.61	1.89	0.17	0.30
SOL Muscle Mass	0.0016	0.0012^{M}	0.2067	0.1308	0.3525	1.99	0.51	0.71	0.43
EDL Muscle Mass	<0.0001	0.0015^{M}	0.1143	0.0001^{M}	0.0011^{M}	1.81	0.67	2.28	1.89
SOL L_0	0.0962	0.2564	0.0656	0.6007	0.6394	0.56	0.72	0.24	0.00
EDL L_0	0.2221	0.0200	0.0124	0.9592	0.0497	1.24	1.19	0.02	0.97
SOL CSA	0.0017	0.0082^{M}	0.3928	0.1471	0.2930	1.46	0.38	0.69	0.58
EDL CSA	<0.0001	0.0082^{M}	0.5792	<0.0001^{M}	0.0086^{M}	1.45	0.24	2.37	1.99
SOL Slow MHC	0.0571	N/A	0.0981	N/A	0.3970	N/A	0.99	N/A	0.49
EDL Slow MHC	0.9334	N/A	0.2764	N/A	0.3182	N/A	0.60	N/A	0.63
DIA Slow MHC	0.8821	N/A	0.1563	N/A	0.7946	N/A	0.72	N/A	0.15
SOL Fast MHC	0.1196	N/A	0.2512	N/A	0.3057	N/A	0.66	N/A	0.57
EDL Fast MHC	0.1120	N/A	0.1380	N/A	0.4582	N/A	0.85	N/A	0.33
DIA Fast MHC	0.3942	N/A	0.8333	N/A	0.3186	N/A	0.12	N/A	0.56

SOL Slow/Fast MHC	0.6458	N/A	0.3566	N/A	0.8342	N/A	0.52	N/A	0.13
EDL Slow/Fast MHC	0.1235	N/A	0.7268	N/A	0.0381	N/A	0.18	N/A	1.25
DIA Slow/Fast MHC	0.0476	N/A	0.1800	N/A	0.1425	N/A	0.77	N/A	0.85
					Isometrics				
SOL Twitch Force	0.0072	0.5896	0.0065[a]	0.0940	0.9211	0.26	1.32	0.95	0.05
EDL Twitch Force	0.0027	0.0039[M]	0.1274	0.1339	0.2531	1.62	0.68	0.72	0.57
SOL Twitch Stress	0.9453	0.0227	0.0236	0.7154	0.2414	1.21	1.05	0.18	0.17
EDL Twitch Stress	0.4942	0.0231	0.1869	0.8752	0.2122	1.19	0.58	0.07	0.61
DIA Twitch Stress	0.5862	0.3541	0.3185	0.9352	0.3439	0.44	0.44	0.04	0.48
SOL Tetanus Force	0.5235	0.0128	0.3351	0.6887	0.0625	1.35	0.42	0.18	0.96
EDL Tetanus Force	0.2045	0.1076	0.3182	0.1847	0.7364	0.81	0.44	0.63	0.16
SOL Tetanus Stress	0.1116	0.6062	0.5867	0.5220	0.0012	0.25	0.24	0.30	1.82
EDL Tetanus Stress	0.0155	0.6688	0.1530	0.1806	0.0484[F]	0.32	0.64	0.63	1.02
DIA Tetanus Stress	0.1307	0.3297	0.4038	0.8576	0.1562	0.48	0.37	0.08	0.76
SOL THPT	0.0015	0.7495	0.0072[F]	0.1357	0.0461[F]	0.17	1.21	0.64	0.84
EDL THPT	0.0031	0.0234[F]	<0.0001[F]	0.9235	0.3392	1.42	3.03	0.00	0.47
DIA THPT	0.2137	0.1203	0.6645	0.1927	0.5123	0.84	0.00	0.63	0.26

SOL LSHR	0.0080	0.0449[M]	0.2654	0.2396	0.3673	0.95	0.51	0.54	0.41
EDL LSHR	0.7257	0.6078	0.8089	0.3221	0.5754	0.00	0.00	0.48	0.37
DIA LSHR	0.8841	0.1309	0.3297	0.8055	0.0928	0.95	0.48	0.19	0.84
Work Loop Power Output-Cycle Frequency									
SOL Absolute Power	<0.0001	<0.0001[M]	<0.0001[M]	<0.0001[M]	0.9556	2.38	0.68	0.69	0.01
EDL Absolute Power	<0.0001	<0.0001[M]	<0.0001[F]	<0.0001[M]	<0.0001[M]	2.61	0.57	0.68	0.82
SOL PO.BM	<0.0001	<0.0001[M]	0.0445[F]	<0.0001[M]	0.0497[F]	0.88	0.18	0.90	0.30
EDL PO.BM	0.5414	0.4053	<0.0001	<0.0001	0.0006	0.15	0.84	0.63	0.86
SOL Normalised Power	<0.0001	0.0003[M]	0.0004[M]	0.0198[M]	0.0010[F]	0.59	0.40	0.24	0.40
EDL Normalised Power	<0.0001	0.9719	<0.0001[F]	0.6028	0.0341[F]	0.01	0.80	0.06	0.22
DIA Normalised Power	0.0132	0.0159[M]	0.0176[F]	0.0617	<0.0001[F]	0.40	0.35	0.23	0.36
Fatigue Resistance									
SOL Fatigue Time	0.0058	0.5594	0.0430[F]	0.4033	0.0479[F]	0.24	0.89	0.39	1.00
EDL Fatigue Time	0.3758	0.0351	0.4073	0.0713	0.0159	0.90	0.43	0.58	1.11
DIA Fatigue Time	0.2057	0.0518	0.1085	0.7143	0.2318	1.16	0.81	0.22	0.65

Table S1: A list of all the computed p-values for male and female CD-1 mouse body mass, muscle morphology, isometrics and the work loop power output-cycle frequency (PO-CF) relationship for soleus, EDL and diaphragm, with sex set as the main effect. All variables tested using ANOVA's (two-way ANOVA –

morphology, isometrics & fatigue resistance; three-way ANOVA – work loop PO-CF) with Tukey's post-hoc analysis used to compare the dependent variables of each sex at a specific age. Values reported to 2 decimal places/significant figures.

SOL, soleus; EDL; extensor digitorum longus; DIA, diaphragm; MHC, myosin heavy chain; L_0, optimal muscle length CSA, cross-sectional area; THPT, time to half-peak tetanus; LSHR, last stimulus to half-relaxation.

[M] Mean value for the given variable is significantly greater for males than females.

[F] Mean value for the given variable is significantly greater for females than males.

		P-Values					
		3 weeks vs.			**10 weeks vs.**		**52 weeks vs.**
ariable	**Main Effect (Age)**	**10 weeks**	**52 weeks**	**78 weeks**	**52 weeks**	**78 weeks**	**78 weeks**
				Morphology			
ody Mass	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0641
DL Muscle Mass	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0023	0.3486
DL Muscle Mass	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.2647	0.0044
DL L_o	<0.0001	<0.0001	<0.0001	<0.0001	0.9186	0.9949	0.9837
DL L_o	<0.0001	<0.0001	<0.0001	<0.0001	0.9095	0.3591	0.7295
DL CSA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0009	0.4184
DL CSA	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.6672	0.0024
				Isometrics			
DL Twitch Force	<0.0001	<0.0001	<0.0001	<0.0001	0.0905	0.8532	0.4522
DL Twitch Force	<0.0001	<0.0001	<0.0001	<0.0001	0.9923	0.3669	0.2435
DL Twitch Stress	<0.0001	0.5852	0.8716	0.7824	0.1569	0.1271	0.9955
DL Twitch Stress	<0.0001	<0.0001	0.0212	0.0291	0.0426	0.0645	1.0000
A Twitch Stress	<0.0001	0.0834	0.3934	0.9616	0.0008	0.0262	0.7000

)L Tetanus Force	<0.0001	<0.0001	<0.0001	<0.0001	0.7312	0.6891	0.9992
)L Tetanus Force	<0.0001	<0.0001	<0.0001	0.0023	0.9728	0.0034	0.0099
)L Tetanus Stress	<0.0001	<0.0001	0.0154	0.0004	0.0001	0.0172	0.4084
)L Tetanus Stress	<0.0001	0.0008	0.9072	0.9899	0.0026	0.0003	0.7539
A Tetanus Stress	<0.0001	0.0015	0.6009	0.4584	<0.0001	<0.0001	0.9889
)L THPT	0.0021	0.3101	0.0267	0.0142	0.5751	0.3658	0.9722
)L THPT	0.1443	0.9930	0.9930	0.2407	0.9397	0.1213	0.3144
A THPT	0.1337	0.9708	0.5163	0.9905	0.2322	0.9993	0.3391
)L LSHR	0.0297	0.8008	0.9842	0.6823	0.5446	0.9931	0.4303
)L LSHR	<0.0001	0.9165	0.8553	0.0273	0.4323	0.0035	0.1115
A LSHR	0.0003	0.0533	0.0415	<0.0001	0.9994	0.0471	0.0603
			Work Loop Power Output-Cycle Frequency				
)L Absolute Power	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.8005	<0.0001
)L Absolute Power	<0.0001	<0.0001	<0.0001	<0.0001	0.0017	<0.0001	<0.0001
)L PO.BM	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0005
)L PO.BM	<0.0001	<0.0001	0.4047	<0.0001	<0.0001	<0.0001	<0.0001
)L Normalised Power	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0004

)L Normalised Power	<0.0001	<0.0001	0.1158	<0.0001	<0.0001	<0.0001	<0.0001
A Normalised Power	<0.0001	<0.0001	0.8332	0.0052	<0.0001	<0.0001	0.0385
				Fatigue Resistance			
)L Fatigue Time	0.0058	0.0210	0.0059	0.0333	0.9508	1.0000	0.9494
)L Fatigue Time	<0.0001	0.9922	0.0031	0.6885	0.0037	0.8121	0.0561
A Fatigue Time	<0.0001	0.0107	<0.0001	<0.0001	0.0525	0.0011	0.3612

Table S2: A list of all the computed p-values for female CD-1 mouse body mass and muscle morphology, isometrics and work loop power output-cycle frequency (PO-CF) relationship for soleus, EDL and diaphragm with age set as the main effect. All variables tested using ANOVA's (two-way ANOVA – morphology, isometrics & fatigue resistance; three-way ANOVA - PO-CF) with Tukey's post-hoc analysis used to compare the dependent variables of between each age for females.

SOL, soleus; EDL; extensor digitorum longus; DIA, diaphragm; L_0, optimal muscle length; CSA, cross-sectional area; THPT, time to half-peak tetanus; LSHR, last stimulus to half-relaxation; PO.BM, power output relative to body mass.

		P-Values									
		3 weeks vs.				**10 weeks vs.**			**30 weeks vs.**		**52 weeks vs.**
	Main Effect (Age)	**10 weeks**	**30 weeks**	**52 weeks**	**78 weeks**	**30 weeks**	**52 weeks**	**78 weeks**	**52 weeks**	**78 weeks**	**78 weeks**
						Morphology					
ass	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.8241	0.9312	0.9992
scle Mass	<0.0001	0.0005	<0.0001	<0.0001	<0.0001	0.0003	<0.0001	0.0104	0.9111	0.7849	0.2887
scle Mass	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0944	<0.0001	<0.0001	0.0003	0.1067	0.1823
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.9970	0.8508	0.2720	0.9686	0.5036	0.8662
	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.9990	0.7348	0.0815	0.8883	0.1786	0.6628
	<0.0001	0.2536	<0.0001	<0.0001	<0.0001	0.0006	<0.0001	0.0028	0.8194	0.9861	0.5190
	<0.0001	0.0195	<0.0001	<0.0001	<0.0001	0.1109	<0.0001	<0.0001	0.0001	0.0080	0.5976
						Isometrics					
tch Force	<0.0001	<0.0001	<0.0001	<0.0001	0.0034	0.7689	0.9983	0.1066	0.9198	0.0100	0.0773
tch Force	<0.0001	0.0019	0.0026	0.0002	0.0476	0.9999	0.8328	0.8235	0.8948	0.8055	0.2858
tch Stress	<0.0001	<0.0001	0.0122	0.3264	0.9917	0.0274	0.0003	<0.0001	0.5593	0.0374	0.5853
tch Stress	<0.0001	0.0115	0.2889	0.9892	0.9872	0.6609	0.0406	0.0027	0.5565	0.1146	0.8663
tch Stress	<0.0001	0.1229	0.3754	0.9849	0.7227	0.9828	0.0365	0.8733	0.1553	0.9918	0.4297

anus Force	<0.0001	<0.0001	<0.0001	<0.0001	0.0003	0.2056	0.9930	0.2738	0.4597	0.0025	0.1603
anus Force	<0.0001	0.0008	0.0005	<0.0001	0.1703	0.9949	0.4751	0.2867	0.7531	0.1788	0.0112
anus Stress	<0.0001	<0.0001	0.0008	0.3609	0.9801	0.0197	<0.0001	<0.0001	0.1060	0.0042	0.6975
anus Stress	<0.0001	0.0203	0.4481	1.0000	0.2046	0.6031	0.0165	<0.0001	0.4031	0.0035	0.2350
anus Stress	<0.0001	0.0065	0.6064	0.2628	0.9107	0.2176	<0.0001	0.0012	0.0108	0.2071	0.8327
T	0.0021	0.9997	0.9883	0.6878	0.2486	0.9564	0.5230	0.1435	0.9216	0.5074	0.9361
T	0.1443	0.1675	0.9994	0.2648	0.7860	0.2582	0.0007	0.0102	0.1763	0.6536	0.8914
T	0.1337	0.2310	0.1510	0.2275	0.1557	0.9963	1.0000	0.9873	0.9994	0.9998	0.9961
R	0.0297	0.2601	0.4645	0.9997	0.1348	0.9982	0.3554	0.9883	0.5790	0.9451	0.1928
R	<0.0001	0.8391	0.9978	0.9847	0.1709	0.6558	0.9885	0.0118	0.9186	0.2964	0.0569
R	0.0003	0.9988	0.5046	0.7107	0.5144	0.6172	0.8199	0.6222	0.9972	1.0000	0.9936

Work Loop Power Output-Cycle Frequency

olute Power	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	0.0007	<0.0001	<0.0001
olute Power	<0.0001	<0.0001	<0.0001	<0.00001	<0.001	0.1370	<0.0001	0.9943	<0.0001	0.3573	<0.0001
BM	<0.0001	<0.0001	<0.0001	<0.0001	0.0016	0.1413	0.2580	<0.0001	0.0005	<0.0001	<0.0001
BM	<0.0001	0.0914	0.0338	0.0003	0.0001	<0.0001	0.2753	<0.0001	<0.0001	0.5050	<0.0001
malised Power	<0.0001	<0.0001	<0.0001	<0.0001	0.1056	0.4180	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001

malised Power	<0.0001	<0.0001	0.9982	0.1665	<0.0001	<0.0001	<0.0001	0.1853	0.0837	<0.0001	<0.0001
malised Power	<0.0001	0.6116	0.7550	<0.0001	<0.0001	0.9998	<0.0001	<0.0001	<0.001	<0.0001	0.0028
						Fatigue Resistance					
gue Time	0.0058	<0.0001	<0.0001	<0.0001	0.0001	0.9993	1.0000	0.6634	0.9999	0.5659	0.6442
gue Time	<0.0001	0.0013	<0.0001	<0.0001	<0.0001	0.1975	0.0011	0.0458	0.3011	0.9644	0.6855
gue Time	<0.0001	0.6153	0.7635	0.1517	0.4633	0.9997	0.8266	0.99	0.7635	0.9769	0.9859

Table S3: A list of all the computed p-values for male CD-1 mouse body mass and muscle morphology, isometrics and work loop power output-cycle frequency (PO-CF) relationship for soleus, EDL and diaphragm with age set as the main effect. All variables tested using ANOVA's (two-way ANOVA – morphology, isometrics & fatigue resistance; three-way ANOVA - PO-CF) with Tukey's post-hoc analysis used to compare the dependent variables of between each age for males. Values reported to 2 decimal places/significant figures.

SOL, soleus; EDL; extensor digitorum longus; DIA, diaphragm; MHC, myosin heavy chain; L_0, optimal muscle length; CSA, cross-sectional area; THPT, time to half-peak tetanus; LSHR, last stimulus to half-relaxation; PO.BM, power output relative to body mass.