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THE EFFECTS OF 8 WEEKS VOLUNTARY WHEEL RUNNING ON THE CONTRACTILE PERFORMANCE OF ISOLATED LOCOMOTORY (SOLEUS) AND RESPIRATORY (DIAPHRAGM) SKELETAL MUSCLE DURING EARLY AGEING

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ABSTRACT

Decreased skeletal muscle performance with increasing age is strongly associated with reduced mobility and quality of life. Increased physical activity is a widely prescribed method of reducing the detrimental effects of ageing on skeletal muscle contractility. The present study uses isometric and work loop testing protocols to uniquely investigate the effects of 8 weeks of voluntary wheel running on the contractile performance of isolated dynapenic soleus and diaphragm muscles of 38 week old CD1 mice. When compared to untrained controls, voluntary wheel running induced significant improvements in maximal isometric stress and work loop power, a reduced resistance to fatigue, but greater cumulative work during fatiguing work loop contractions in isolated muscle. These differences occurred without appreciable changes in LDH, CS, SERCA or MHC expression synonymous with this form of training in younger rodent models. Despite the given improvement in contractile performance, the average running distance significantly declined over the course of the training period, indicating that this form of training may not be sufficient to fully counteract the longer term ageing induced decline in skeletal muscle contractile performance. Although these results indicate that regular low intensity physical activity may be beneficial in offsetting the age-related decline in skeletal muscle contractility, the present findings infer that future work focusing on the maintenance of a healthy body mass with increasing age and its effects on myosin-actin cross bridge kinetics and Ca^{2+} handling, is needed to clarify the mechanisms causing the improved contractile performance in trained dynapenic skeletal muscle.

KEY WORDS: Dynapenia, Training, Contractile Performance, Weight Management, Sarcopenia

INTRODUCTION

An age-related reduction in mobility and quality of life is associated with a decrease in the contractile performance of skeletal muscle (Williams et al., 2002, Landi et al., 2012, Woo et al., 2016). As such, older adults are encouraged to engage in regular physical activity to offset degenerative changes in muscle contractility (WHO, 2017, Marcell, 2003, Iolascon et al., 2014). Although *in vivo* evidence is rife with studies reporting training induced improvements in the maximal strength and power of older adults (Pyka et al., 1994, McCartney et al., 1995, Mayer et al., 2011, Melov et al., 2007, Seguin and Nelson, 2003, Reid et al., 2008, Reid et al., 2015), fewer studies have examined training responses during the onset of muscle ageing, which has been shown to occur relatively early on in life (Lexell, 1995). Given that mechanistically the age associated decline in muscle contractility has been attributed to processes both integral to the muscle and the wider body (Doherty, 2003), work is needed to distinguish the effect of training directly on skeletal muscle. The present study uses an *in vitro* isolated skeletal muscle approach to examine the effects of voluntary wheel running on muscle contractility in rodents at the early stages of the muscle ageing process.

Previous work examining isolated skeletal muscle contractility in aged rodent models (Brooks and Faulkner, 1988, Gonzalez et al., 2000, Zhang and Kelsen, 1990) has provided a vital contribution to our understanding of the muscle ageing response. Only very recently however, have such models been employed to investigate the effects of early ageing on muscle contractility (Tallis et al., 2014b). Work by our research group using an inbred strain of female CD-1 mice has demonstrated that contractile performance has significantly declined by 30 weeks of age. There is a distinct dearth of evidence exploring the effect of training on skeletal muscle plasticity during early ageing; having a better understanding of the training adaptations that occur at this time may prove important for understanding and reducing the more severe reduction in muscle contractility that occurs during the onset of sarcopenia in older ageing.

Examining training-induced changes in the contractile performance of isolated skeletal muscle allows muscle specific changes in contractility to be determined. *In vivo* assessments of muscle function are largely performed using gross motor skills, and consequently, require the recruitment of muscle groups. Due to variations in cross sectional area, architecture and fibre type composition, it is difficult to ascertain the muscle-specific contractile response. Furthermore, the sequence of electrical (central nervous system) and mechanical processes involved with force production make it difficult to determine the magnitude of any training-induced benefits at the muscle level. Previous *in vivo* work, examining training effects on muscle contractility, report gains in absolute muscle force and power (Pyka et al., 1994, McCartney et al., 1995, Mayer et al., 2011, Melov et al., 2007, Seguin and Nelson, 2003, Reid et al., 2008, Reid et al., 2015), which tells us very little about muscle contractility relative to size. Importantly, assessment of contractile performance using isolated muscle allows muscle quality to be determined (measure of force or power relative to muscle size, Tallis et al., 2017). A training-induced increase in muscle quality would be more desirable than an increase in muscle mass. Muscle producing high force and power in a lower quantity of tissue (i.e. good muscle quality) would reduce tissue maintenance cost and body mass, thus reducing the force that must be produced by the musculature to overcome inertia of the body.

Irrespective of age, studies that have examined skeletal muscle adaptions to training have primarily focused on results from biochemical analysis to quantify changes in fibre type composition, metabolic capacity and vascular adaptations (Brown et al., 1992, Sullivan et al., 1995, Houle-Leroy et al., 2000, Allen et al., 2001, Behnke et al., 2012). Surprisingly, only a very small number of studies have examined the effect of physical training on isolated skeletal muscle contractility, with equivocal findings (Taylor et al., 1976, Metzger and Fitts, 1986, Carter et al., 1995, Zhan et al., 1999, Ergen et al., 2005, Willems and Stauber, 2000, Hayes and Williams, 1996). The ambiguity in research findings can likely be attributed to differences in gender, age, strain and species of animals, method and duration of training, and experimental protocol used to measure skeletal muscle contractility.

Such previous assessments of isolated muscle function examine contractility using isometric and isovelocity methods which have poor relevance to *in vivo* muscle function where cyclical power producing contractions are performed (Josephson, 1985, James et al., 1995). As such, the present study will use the work loop technique to uniquely examine the effect of training using a methodological approach that better approximates *in vivo* muscle function (Josephson, 1985). Like *in vivo* contractile function, this method considers the interaction between force produced during shortening, passive resistance to stretch, and influence of activation and relaxation rates, using waveforms and stimulation patterns that more closely replicate *in vivo* patterns (James et al., 1995, James et al., 1996, Tallis et al., 2014b, Josephson, 1985). Other assessments of muscle contractility fail to consider the interaction of these important mechanical characteristics, and a change in one or a combination of these factors could have profound effects on peak power and fatigue resistance that may not be demonstrated accurately using other methods. Furthermore, previous assessments of training-induced changes in muscle power have been derived from isovelocity assessments of muscle contractility (Zhan et al., 1999) which substantially overestimate the power output measured by the work loop technique (James et al., 1996).

The aim of the present study was to examine the effects of voluntary wheel running on the contractile properties of soleus and diaphragm muscle isolated from 38 week old CD-1 mice. Given that previous work has demonstrated that the onset of muscle ageing occurs by 30 weeks of age in these muscles, for this strain of mice (Tallis et al., 2014b), the present work offers an important insight into skeletal muscle plasticity during the initial stages of the ageing process. The soleus muscle has an important role in locomotion (James et al., 1995, Nicolopoulos-Stournaras and Iles, 1984) and postural control. Voluntary wheel running has been demonstrated to increase the proportion of slow oxidative fibres in young mice (Allen et al., 2001), and promote hypertrophy of the soleus in both young and old rats (Brown et al., 1992). Despite this evidence, it is not clear how voluntary wheel running will directly affect soleus contractility. Furthermore, there is a wealth of literature outlining the benefits of low intensity,

long duration exercise training on ventilatory performance (Sheel, 2002). However given the importance of the diaphragm muscle in pulmonary function, there is a dearth of research that considers the effects of training on the contractile function of this muscle (Sheel, 2002). It was hypothesised that voluntary wheel running would evoke improved force, power and fatigue resistance of both the soleus and diaphragm, which would be underpinned by a greater muscle mass, shift to a slower fibre type and an increased oxidative capacity.

Method

Animals & Training

The ethics committee of Coventry University approved the use of animals in this study. Female white mice (strain CD1, Charles River, UK) were bred and kept in house at Coventry University. From birth, and throughout the duration of the experiment, animals had *ad libitum* access to food (CRM(P); SDS/Dietex International Ltd) and water, and were kept in 12:12 light:dark cycles at 50% relative humidity. For the first 30 weeks animals were kept in groups of 8 without access to running wheels. Previous work by our group has indicated that the greatest early ageing related decline in skeletal muscle contractile performance in female CD1 mice occurs between 30-50 weeks (Tallis et al., 2014b), and we therefore used 38 week old mice to examine the effects of training on the early ageing response. At 30 weeks of age, animals were weighed to the nearest 0.1 g and randomly divided into a training group and a control group (n=14 in each group; body mass 45±2g and 43±1g for training and control group respectively; t-test p=0.23). For the duration of the experiment, mice in both the control and treatment groups were housed in individual cages. Each mouse in the training group had access to a running wheel (diameter = 15cm) for 8 weeks, with revolutions recorded either every 30 minutes (n= 7) or every 24 hours (n= 7). We monitored the activity of a subset of training group animals at 30 minute intervals to more closely determine the daily variation in activity. Running wheels were locked 24-48 hours prior to assessment of the contractile and biochemical properties of the target skeletal muscle. For the duration of the experiments control animals were housed in identical conditions without access to running wheels.

Dissection

The dissection procedure and assessment of mechanical properties were carried out according to published protocols (James et al., 2005, Higgins et al., 2013, Tallis et al., 2012, Tallis et al.,

2014b). Animals were sacrificed by cervical dislocation, in accordance with British Home Office Animals (Scientific Procedures) Act 1986, Schedule 1, and then weighed to determine body mass. Both hind limbs or the thoracic cavity were rapidly removed from the animal at room temperature (19-21°C), and throughout the dissection process were kept in regularly changed, cooled (1-4°C), oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit solution (composition in mM: NaCl 118; KCl 4.75; $MgSO_4$ 1.18; $NaHCO_3$ 24.8; KH_2PO_4 1.18; glucose 10; $CaCl_2$ 2.54; pH 7.55, at room temperature prior to oxygenation). Soleus muscle was used to serve as an indicator of locomotor muscle adaptations and diaphragm as an indicator of changes in pulmonary function.

Soleus muscle from the left hind limb was isolated and frozen immediately in liquid nitrogen, then stored in a -80°C freezer for later biochemical analysis. Soleus muscle from the right hind limb was then isolated and pinned out at approximately its resting length, and aluminium foil T clips were placed around both the proximal and distal tendons. The T-clips were used to prevent tendon slippage during muscle activation and allow attachment of the muscle preparation to the custom designed muscle rig. Whole diaphragm muscle was dissected, but only a ventral section of the costal diaphragm was used in the protocol to analyse contractile performance of muscle. Aluminium foil T-clips were wrapped around the central tendon at one end, and at the opposing end two ribs anchoring the muscle were left intact, and the T-clip and the ribs were used to attach the muscle preparation to the muscle rig. The left half of the diaphragm was dissected and frozen in liquid nitrogen.

Assessment of Contractile Properties

One end of each muscle was attached to a force transducer (UF1, Pioden Controls), and the opposite end to a motor arm (V201, Ling Dynamic Systems) to control length changes. Position of the motor arm was determined by a Linear Variable Displacement Transformer (DFG5.0, Solartron Metrology). Temperature in the muscle chamber was maintained at 37 ± 0.2°C

224 throughout the duration of the protocol. Prior to assessment of contractile properties, each
225 muscle preparation was left in the muscle chamber for 10 minutes to equilibrate to the new
226 environment. Initially each muscle was subjected to a series of isometric twitches while
227 stimulation amplitude (usually 12-16 V & 10-16V for soleus & diaphragm respectively: current
228 fixed at 160mA) and length were optimised to produce a maximal twitch force response.
229
230 At the predetermined optimal length for twitch force, each muscle preparation was subjected to
231 a series of stimulations, and stimulation frequency was optimised (usually 140Hz for both
232 soleus & diaphragm) at a fixed burst duration (350 ms & 250 ms for soleus & diaphragm
233 respectively) to evoke maximal tetanic force. Time to half peak tetanus (THPT) and time from
234 last stimulus to half tetanus relaxation (LSHR) were measured as indicators of muscle
235 activation and relaxation time, respectively. The muscle length that corresponded to maximal
236 isometric stress was measured using an eyepiece graticule fitted to a dissection microscope, and
237 was defined as L_0. 85% of the measured muscle length was used as an estimate of fibre length
238 for soleus, in line with previous research (James et al., 1995). No such estimates of fibre length
239 have been reported for diaphragm so the physical length measured was used as L_0, as in
240 previous work (Tallis et al., 2014b). An interval of 5 minutes was given between tetanic
241 stimulations in order to allow sufficient muscle recovery time.
242
243 Muscle power output was assessed using the work loop technique. Here each muscle was
244 subjected to a symmetrical sinusoidal length change waveform around the previously
245 determined L_0. A typical strain of 0.10 was used and the muscle was electrically stimulated
246 during shortening using the optimised stimulation amplitude and frequency parameters that
247 yielded maximal isometric force. Put simply, the muscle was stretched by 5% of L_0 whilst
248 passive, then shortened by 10%, and was then lengthened by 5% back to L_0. Instantaneous
249 power output was calculated for every data point in each work loop (10,000 data points per
250 work loop) by multiplying instantaneous velocity by instantaneous force. Instantaneous power

output values were averaged across the entire length change cycle to generate an average power output for each length change cycle.

A cycle frequency of 5Hz and 7Hz was used for soleus and diaphragm muscle, respectively, concurrent with previous research which found that these cycle frequencies elicit maximal power output in these muscles (Altringham and Young, 1991, James et al., 1995, Tallis et al., 2012, Askew and Marsh, 1997). The cycle frequencies chosen have been found to elicit maximal power output in mice of this age and strain (authors, unpublished work). Furthermore, these cycle frequencies are attainable *in vivo* (James et al., 1995). The strain of 0.10 was based on previous estimations of the strain required for production of maximal power in both soleus and diaphragm (James et al., 2005, Altringham and Young, 1991). Stimulation parameters were then adjusted to optimise net work. The duration of electrical stimulation during the shortening phase was further optimised to evoke maximal net work. 65 and 55 ms burst durations were used for soleus and diaphragm, respectively, as described in previous work (James et al., 1995, James et al., 1996, Tallis et al., 2014a). On occasions the burst duration had to be altered to adjust the number of stimuli given in order to maximise muscle power output of individual muscle preparations. The alteration in stimulation duration was determined by examining the maximal work loop power output and by interpretation of the work loop shapes. A stimulation phase shift, which is the period of time before the onset of the shortening that stimulation begins, of -10ms and -5ms was used for soleus and diaphragm, respectively, in order to elicit maximal net work (Tallis et al., 2014b).

The magnitude and frequency of length changes and electrical stimulation were controlled via custom written software (Testpoint, CEC) via a D/A board (KPCI3108, Keithley Instruments). Each muscle was subjected to a set of four sinusoidal length change cycles at 10-minute intervals until maximal muscle power output was achieved. The third work loop, of each set of four, typically produced the highest power and was therefore taken as the indicative measure of muscle power output in all work loop experiments.

Fatigue of muscle power output was tested by subjecting the muscle to 100 consecutive work loops at the previously determined optimal parameters for maximal power output. Power output was recorded for every second work loop, and the time for power to fall below 50% of the pre-fatigue maximum was used to indicate fatigue resistance. Cumulative net work was calculated across the fatigue run for each experimental group as the sum of the mean work produced during every second work loop until the muscle was producing less than 50% of the maximal power. Recovery of maximal muscle power output was recorded for 30 minutes, by subjecting the muscle to a set of four sinusoidal length change cycles at 10 minute intervals.

The experimental protocol was 190 minutes in duration, and control runs were performed regularly to monitor muscle contractile performance over time. After 150 minutes, at the start of the fatigue test, muscle power output had declined by 11% of its maximal value in both soleus and diaphragm. A set of control stimulation and length change parameters were repeated throughout the duration of the experimental procedure to allow all power output values to be corrected to avoid the small decline in muscle quality over time confounding the overall results of the study.

At the end of these assessments the foil clip, bone and tendons were removed and the remaining muscle blotted on absorbent paper and placed on an electronic balance (Mettler-Toledo B204-S, Greifensee, Switzerland) to determine wet muscle mass to the nearest 0.0001 g. Mean muscle cross-sectional area was calculated from muscle length and mass assuming a density of 1060 kg m^{-3} (Mendez and Keys 1960). Maximum isometric muscle stress was calculated as maximal tetanic force divided by mean muscle cross-sectional area (kN m^{-2}). Normalised muscle power output was calculated as power output divided by wet muscle mass (W kg^{-1}).

Biochemical analysis

We measured activity of lactate dehydrogenase as an indictor of glycolytic capacity, and citrate synthase activity as an indicator of mitochondrial density and oxidative capacity (Larsen et al.,

2012). Enzyme activities were determined (in n = 8 animals per treatment) according to published protocols (Seebacher et al., 2003). We determined the activity of sarco-endoplasmic reticulum ATPase (SERCA) as a biochemical indicator of muscle contractile function (in n = 8-9 animals per treatment). SERCA re-sequesters Ca^{2+} into the sarcoplasmic reticulum to initiate muscle relaxation so that its activity is related to muscle contractile function (Berchtold et al., 2000). SERCA activity was determined according to published protocols (James et al., 2011).

Concentrations of myosin heavy chain fast and slow isoforms were determined by capillary electrophoresis in a "Wes" Simple Western system (Protein Simple, Santa Clara, CA, USA) according to the manufacturer's instructions (in n = 5 animals per treatment). We used anti-fast (ab51263) and anti-slow (ab11083) skeletal myosin heavy chain antibodies, and α-tubulin (ab80779) as internal control (Lee et al., 2012) (all from Abcam, Cambridge, MA, USA).

Statistical Analysis

Mean distance covered during each week of the training protocol was compared between training weeks using a repeated measures ANOVA, with training week as the fixed factor. Pairwise comparisons with Bonferroni correction were used to determine where specific differences occurred. Independent samples t tests were used to examine statistical significance between treatments in whole animal body mass and muscle mass post the treatment intervention. A series of Muscle (2) X Treatment (2) ANOVAs were used to examine the training effect on isometric twitch stress, tetanus stress, maximal work loop power, fatigue, recovery post fatigue, and enzyme activities. Interaction effects were used to determine if the training response was muscle specific. As cumulative work was measured up until the muscle was producing < 50% of its pre fatigue maximal power, the effect of training on this measure was analysed by independent samples t test. For both the trained muscle groups, Pearson's

correlation tests were performed to determine the relationship between training volume and muscle contractile performance (i.e tetanus stress, work loop power and time to fatigue).

We used a three-way ANOVA to test for differences in myosin heavy chain concentrations with Muscle (2) X Treatment (2) X Myosin Isoform (2) as fixed factors. Interaction effects were reported to determine if the training response was muscle specific. All statistical analysis was performed using SPSS (Version 22, SPSS) and significance was determined when $P<0.05$. Data is represented as mean ± SE.

The truncated product method (Zaykin et al., 2002) was used to analyse the distribution of *P*-values in this study to provide a P value for each group of multiple hypothesis tests to assess the P-values were biased via multiple hypothesis testing. The truncated product method *P*-value was 0.0013, for the *Wheel Running, Body Mass & Muscle Mass* group of P values, and <0.001, for the *Skeletal Muscle Contractility* group of P values, indicating that statistical results were not skewed by multiple hypothesis testing.

RESULTS

Wheel Running, Body Mass & Muscle Mass

The average weekly distance covered was significantly affected by time (Fig 1. ANOVA $p < 0.001$). Distance covered in week one was significantly lower than in all other weeks (Fig 1. Bonferroni $p < 0.02$ in all cases). The maximal distance of 5.97 ± 0.29 km was achieved at week two and declined thereafter but was only statistically lower at week 8 (Fig 1; 4.96 ± 0.31m; Bonferroni $p=0.009$).

Post treatment, whole animal body mass of the control group was substantially greater compared to the trained group (52±6g and 41±1g for control and trained group respectively; t-test; $p=0.028$). The body mass of the trained group did not change over the duration of the intervention (T-Test $p=0.13$).

Soleus muscle mass was not significantly different between the trained and non-trained group (11±0.08mg & 11±0.07mg respectively; T-Test; $p = 0.94$). As only a section of the diaphragm was taken, which unavoidably differed in size between each extraction, diaphragm mass has not been reported

Skeletal Muscle Contractility

Following 8 weeks of voluntary wheel running, there was a statistical tendency for maximal isometric twitch stress to be greater in the trained group compared to the control group (Fig 2A. ANOVA $p=0.074$). Isometric tetanus stress was significantly greater in trained than control mice in both soleus and diaphragm (Fig 2B. ANOVA $p<0.001$). Time to Half Peak Tetanus (THPT), used as a measure of muscle activation, and Last Stimulus to Half Relaxation (LSHR), used as a measure of relaxation time, were not significantly affected by the training intervention

in either soleus or diaphragm (Fig 3A & B. ANOVA $p>0.38$ in both cases). Following the intervention period, soleus and diaphragm work loop power outputs were significantly higher in the trained group compared to controls (Fig 4. ANOVA $p=0.002$) when expressed as power output per muscle mass.

There was no significant Muscle*Treatment interactions in any of the contractile properties measured (ANOVA $p>0.35$ in all cases).

Fatigue

Both trained soleus and diaphragm muscles fatigued significantly faster (5.31±0.29s vs. 6.00±0.15s and 3.56±0.19s vs. 3.98±0.28s respectively) than their respective controls (Fig 5 A & B. ANOVA $p=0.034$). There was no significant Muscle*Treatment interaction (Fig 5 A & B. ANOVA $p=0.51$). In comparison to their respective control, the cumulative work over the duration of the fatiguing protocol was significantly greater in trained soleus than control soleus (Fig 5 C. T-Test $p<0.001$). Typical work loop shapes indicate that muscles of the trained group had a greater reduction in net work output (indicated by the area of the work loop) over the duration of the fatigue protocol (Fig 6), when compared with controls. The greater reduction in net work output in the trained group is likely due to a reduction in work done during shortening and greater muscle activity during re-lengthening, possibly due to a slowing of relaxation time, contributing to a greater amount of negative work (net work = positive work during shortening – negative work during lengthening).

Soleus and diaphragm recovered to 95% of the pre fatigue maximum within 30 minutes of completion of the fatiguing protocol. Recovery was not significantly different between muscles or treatments (ANOVA $p>0.1$ in both cases).

Biochemical Analysis

No statistically significant Muscle*Treatment interactions were found for LDH, CS, or SERCA activities (Table 2; ANOVA $p>0.5$ in each cases). There was no significant difference between trained and untrained groups for either soleus or diaphragm (Table 2; ANOVA $p>0.2$ in each case).

There was no statistically significant Muscle*Treatment interaction found in myosin heavy chain isoform concentrations (Table 3; ANOVA $p = 0.34$), and no difference between the trained and untrained groups (Table 3; ANOVA $p = 0.51$). The significant Muscle*Myosin Isoform interaction (Table 3: ANOVA $p = 0.04$) shows that diaphragm muscle had greater fast myosin heavy chain concentrations than soleus irrespective of treatment.

DISCUSSION

Skeletal Muscle Contractility

Eight weeks of voluntary wheel running caused a substantial improvement in maximal isometric stress (force normalised to muscle cross-sectional area) and work loop power output (power normalised to muscle mass) of both the soleus and diaphragm muscles in the absence of changes in MHC isoform and metabolic capacity. The present findings are not only the first to demonstrate the value of voluntary wheel running to evoke increased muscle power in isolated muscle, but further indicate the importance of sustained physical activity during early ageing in preventing the age-related increase in body mass and decline in the contractile function of skeletal muscle involved in locomotion or breathing.

The increase in maximal isometric stress aligns with previous training studies using young healthy rodents (Troup et al., 1986, Carter et al., 1995, Hayes and Williams, 1996). However, unlike the present work using an early ageing model, the changes in contractile performance in young rodents were attributed to altered muscle phenotype and metabolic profile. Although the present findings highlight the value of voluntary wheel running as a method for inducing improvements in contractility in skeletal muscle of mixed fibre type, this may not necessarily apply to muscles composed primarily of fast twitch fibres (Carter et al., 1995, Zhan et al., 1999).

While the training-induced increase in maximal isometric stress appeared to be greater in the diaphragm compared to soleus (30% and 23% respectively compared to controls), this did not reach statistical significance as there was no Muscle*Treatment interaction for any of the measured contractile variables. There was little difference in the magnitude of the training induced increase in maximal work loop power between the two muscles (26% & 24% for diaphragm and soleus respectively, when compared to controls). Training induced adaptations likely relate to the intensity and duration of muscle recruitment, and as such, the training

stimulus could be muscle specific. Given that the response was similar for both muscles, these data indicate that either the training stimulus received was equal, or possibly, that plasticity is greater in one of the examined muscles working at a relatively lower intensity. The latter of would be difficult to assess as traditional methods used to indicate muscle activity during exercise, such as electromyography (EMG), would not be appropriate for the diaphragm.

Fatigue Resistance

Previous studies evaluating the effect of training on contractility using young healthy rodent models have demonstrated equivocal findings with respect to fatigue resistance (Metzger and Fitts, 1986, Hayes and Williams, 1996, Zhan et al., 1999, Ergen et al., 2005). Such ambiguity can largely be attributed to differences in the experimental procedures, such as training method and duration, muscle tested and contractile parameter measured, making comparison with prior studies difficult. The present study is, however, the first to examine the effect of training on the fatigability of muscle power in an early ageing or ageing model.

Results of the present work demonstrate that, in comparison to untrained controls, training caused a significant reduction in the ability of both soleus and diaphragm to maintain maximum power output. This may indicate a training induced increase in the force producing capacity of faster fibres within the muscle, and/or a greater disparity between ATP supply and demand given that muscles of the trained group are producing greater force and there are no distinguishable adaptations in regulatory metabolic enzymes. The trained soleus however, produced greater cumulative work over the period of repeated contractions. When considered with respect to *in vivo* function, if both muscles were operating at the same absolute intensity, the trained muscle would have an improved resistance to fatigue by producing the same magnitude of work with a smaller number of recruited fibres, or the same number of fibres working at a relatively lower intensity, due to having an improved maximal power output. With the trained group having a significantly lower body mass compared to controls, the changes in

contractility may be more substantial when considered in relation to whole animal locomotor performance. The muscles of the trained group would be working against a reduced whole body inertia, theoretically resulting in a further increased exercise capacity than would be expected by looking at isolated muscle contractility alone.

The reduction in work done during shortening and increased activation during re-lengthening (as indicated in the typical work loop shapes), over a series of repeated work loop cycles, is indicative of previous studies examining the fatigue response of these muscles using the work loop technique (Tallis et al., 2013, Tallis et al., 2014b). The work loop shapes indicate that these contributors to a reduction in net work, during prolonged cycling of the muscle, were greater in the trained muscles, and thus, may account for the training induced reduction in fatigue resistance.

Mechanisms

Despite the significant improvement in contractile performance, there were surprisingly no significant training induced changes in LDH, CS or SERCA activities, muscle mass or the concentration of fast and slow MHC isoforms in either soleus or diaphragm muscle. This is particularly interesting given the abundance of literature that demonstrates changes in muscle metabolic profile or a shift in fibre type composition as being the primary cause of skeletal muscle adaptations to training (Sullivan et al., 1995, Allen et al., 2001, Sugiura et al., 1992). The prevalence and magnitude of such mechanistic changes are likely to be specific to the muscle studied and the intensity and duration of the training intervention.

In line with the present results, a small number of studies have demonstrated training induced improvements in skeletal muscle contractility in young animal models that have not always been associated with changes in phenotype expression (Taylor et al., 1976, Carter et al., 1995, Zhan et al., 1999). In agreement with the present study, Sullivan et al. (1995) reported that,

training failed to elicit any change in the MHC profile of soleus muscle in young and old rats, and further indicated a muscle and age specific continuum of adaptations relating to exercise intensity.

Although some studies indicate that training induced changes in skeletal muscle metabolic capacity appear to occur much more readily (Allen et al., 2001, Zhan et al., 1999, Ergen et al., 2005), other evidence denotes that this may not always be the case. Allen et al. (2001) reported that 4 weeks voluntary wheel running caused a significant increase in the oxidative capacity of mouse tibialis anterior and a greater expression of type IIa and IId/x fibres. Interestingly the metabolic capacity of gastrocnemius was unchanged and although greater expression of type IIa and IId/x fibres was demonstrated after 2 weeks, this was not maintained after 4 weeks of training.

Much of the evidence outlining training-induced adaptations in fibre type and metabolic capacity comes from studies using healthy young muscle (Allen et al., 2001, Zhan et al., 1999, Ergen et al., 2005) and although there is evidence of a similar method of adaptation in older adults (Menshikova et al., 2006, Flack et al., 2016) this has been explored in less detail. Irrespective of the previous literature, the present data infer that these mechanisms are not the primary cause of training induced adaptations in skeletal muscle contractility that arise following prolonged low intensity exercise in mixed fibre type skeletal muscle undergoing the early stages of age related degeneration. Although the present study demonstrates a substantial increase in muscle contractility, these findings may suggest an age-related reduction in skeletal muscle plasticity which may limit the rate and magnitude of adaptations that can occur during training.

Animals in the training group had a significantly lower body mass than the control group and it may be that a subsequent infiltration of lipid into the muscle of control animals, may contribute to the difference in contractile performance between the control and trained group.

Recent work has demonstrated a link between muscular lipid accumulation and a reduction in the isometric stress and normalised work loop power of isolated skeletal muscle (Ciapaite et al., 2015, Tallis et al., 2017). Akhmedov and Berdeaux (2013) concluded that excessive accumulation of skeletal muscle lipids affects the ability of muscle to maintain and regenerate contractile proteins. The improvement in the contractile performance of the muscles of the trained group may in part be attributed to the effectiveness of exercise in weight management.

The age related reduction in contractile performance has, in part, previously been attributed to a reduction in the effectiveness of the Ca^{2+} handling process (Larsson and Salviati, 1989). Given that no difference was apparent in the isometric activation and relaxation times or SERCA between the trained and the untrained group, it is unlikely that training was unable to substantially reverse these effects. However, an improvement in the efficiency of cross bridge kinetics may occur independently of changes in Ca^{2+} handling, and this may account for the training induced increase in force and power demonstrated in this study. Future work should look to establish the effects of training on rate limiting enzymes and structural proteins such as myosin light chain, myosin ATPase and troponin isoforms that could influence the ability of muscle to produce force.

Voluntary Wheel Running

Mice in the current study ran a daily average of 5.97 ± 0.29km, which is slightly lower than the 6.8 km reported by Allen et al. (2001) in 10 week old mice. Despite the significant improvement in skeletal muscle contractility in the present study, the distance covered by wheel running mice had significantly decreased during the latter stages of the wheel running intervention. This is particularly interesting given that a positive training response should theoretically promote further running distances. The animals, therefore, may not be at the peak of their 'trained' state after the 8 week intervention. This may arise from a problem with an inability to self-regulate a progressive training program, or more likely the effects of increasing age. These findings

indicate that a training-induced increase in the contractile performance of skeletal muscle cannot fully offset the deterioration in muscle contractility that occurs during the early onset of the ageing process. It would be of interest to compare the current results with those obtained using a protocol of treadmill running, where exercise volume and intensity can be more precisely regulated and could be gradually altered over time.

However, it is interesting to note that there was no relationship between training volume and muscle function in either the soleus or diaphragm muscles. However, it is acknowledged that, in future work, individual responses need to be analysed using a larger sample size.

Limitations

Although the work loop technique provides a better approximation of real world muscle function, the length change waveforms used *in vivo* are likely to be more complex than the sinusoidal pattern used in the present study (James et al 1995; Dickinson et al., 2000). Furthermore, the pattern of fibre stimulation and length change waveforms are likely to be manipulated throughout movement (Wakeling and Rozitis, 2005), particularly during repeated contractions in order to minimise the build-up in work done on the muscle during muscle lengthening (Tallis et al., 2013).

The results of the present study offer an important insight into the effects of voluntary wheel running on both the soleus and diaphragm, however these results may not be generalizable to other locomotory muscles. Future work should also consider the effect of voluntary wheel running on hip and knee extensor muscles given the substantial role of such muscle groups in walking and running.

Practical Implications

The present findings show the value of low intensity, high frequency activity on skeletal muscle contractile performance and indicate that such exercise modalities would be beneficial for slowing the loss in skeletal muscle contractility that occurs during early ageing. Importantly, maintaining a greater muscle function during this time may be significant for offsetting the more substantial decline in skeletal muscle contractile performance that occurs during older ageing. The extensive improvement in the contractile performance of the diaphragm is likely to play an important role in the training induced improvement in pulmonary function. Improved pulmonary function could be important for mediating further exercise induced enhancements in muscle contractility by satisfying the increased skeletal muscle oxygen demand, consequently promoting a greater exercise capacity.

Conclusion

The present study demonstrates that eight weeks of voluntary wheel running caused significant improvements in isolated skeletal muscle contractility in mice undergoing early ageing. The results indicate that the increase in muscle contractile performance occurs without significant changes in muscle mass, fibre type or metabolic capacity, in contrast to the findings demonstrated in younger trained muscle. Such findings support the value of physical activity in slowing the early age related decline in muscle contractile performance. Despite the given increase in contractility, the present work infers that voluntary wheel running is not sufficient in fully offsetting the degeneration of muscle contractile performance that occurs during early ageing. Future work focusing on changes in the effectiveness of cross bridge formation and mechanisms related to the obesity associated reduction in muscle contractility may be important in identifying the primary mechanisms causing an improved contractile performance following training during the onset of the skeletal muscle ageing response.

642 **ACKNOWLEDGEMENTS**

643 Thank you to Roy Petticrew, Mark Bodycote & Bethan Grist for technical assistance.

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TABLES

Table 1. Relationship between total wheel running distance and muscle contractile performance

		Maximal Tetanus Stress (kN^{-2})	Work Loop Power (W/kg)	Time to Fatigue (s)
Trained DIA	*R*	0.102	0.102	-0.348
	P	0.827	0.828	0.444
Trained SOL	*R*	0.069	-0.438	-0.379
	P	0.883	0.325	0.401

[N=7 in each case]

Table 2. The effect of 8 weeks of voluntary wheel running on soleus and diaphragm muscle LDH, CS & SERCA activities

	Soleus		Diaphragm	
	Control	*Trained*	*Control*	*Trained*
LDH (µmol mg^{-1} tissue min^{-1})	90.4±10.4	82.4±12.6	149.9±20.5	135.2±15
CS (µmol g^{-1} tissue min^{-1})	23±7	16.8±1.8	87.2±7.4	71.8±12
SERCA (µmol mg^{-1} tissue h^{-1})	1.7±.6	2.87±0.9	6.3±2.5	2.6±0.41

[Data represented as mean ± s.e.; N=7 in each case]

Table 3. The effect of 8 weeks of voluntary wheel running on soleus and diaphragm fast and slow myosin heavy chain concentration (normalized to alpha tubulin)

	Soleus		Diaphragm	
	Control	*Trained*	*Control*	*Trained*
Slow Myosin	0.108±0.026	0.16±0.037	0.1±0.026	0.132±0.032
Fast Myosin	0.063±0.02	0.094±0.052	0.195±0.057	0.148±0.045

[Data represented as mean ± s.e.; N=5 in each case]

728 **FIGURES**

729 Figure 1 – Average daily (white squares) and weekly (black circles and line) mean distance
730 covered during 8 weeks voluntary wheel running in 38 week old CD1 female mice [Data
731 represented as mean ± s.e.; N=14]

732

733 Figure 2 – The effects of 8 weeks voluntary wheel running on the maximal isometric twitch (A)
734 and tetanus stress (force normalised to muscle cross-sectional area) (B) of mouse soleus and
735 diaphragm muscle [Data represented as mean ± s.e.; N=7 in each case; * indicate statistical
736 differences between trained and control groups]

737

738 Figure 3 – The effects of 8 weeks voluntary wheel running on Time to Half Peak Tetanus
739 (THPT; A) and Last Stimulus to Half Relaxation (LSHR; B) in mouse soleus and diaphragm
740 muscle [Data represented as mean ± s.e.; N=7 in each case]

741

742 Figure 4 – The effects of 8 weeks voluntary wheel running on the maximal work loop power
743 output (normalised to muscle mass) of mouse soleus and diaphragm muscle [Data represented
744 as Mean±s.e.; N=7 in each case; * indicate statistical differences between trained and control
745 groups]

746

747 Figure 5 – The effects of 8 weeks voluntary wheel running on the fatigue of muscle power
748 output (A & B) and the cumulative net work during the fatigue protocol (C) in mouse soleus
749 muscle respectively [CS = Control Soleus; TS = Trained Soleus; CD = Control Diaphragm; TD
750 = Trained Diaphragm; Data represented as Mean±s.e.; N=7 in each case]

751

752 Figure 6. Typical work loop shapes for control and trained soleus (A & B) and diaphragm (C
753 & D) [Start of arrow indicates L_0 and direction of lengthening; 0.4s, 2.48, and 5.2s represent
754 time since the start of the fatigue protocol for the soleus; 0.3s, 2.0, and 3.7s represent time
755 since the start of the fatigue protocol for the diaphragm]

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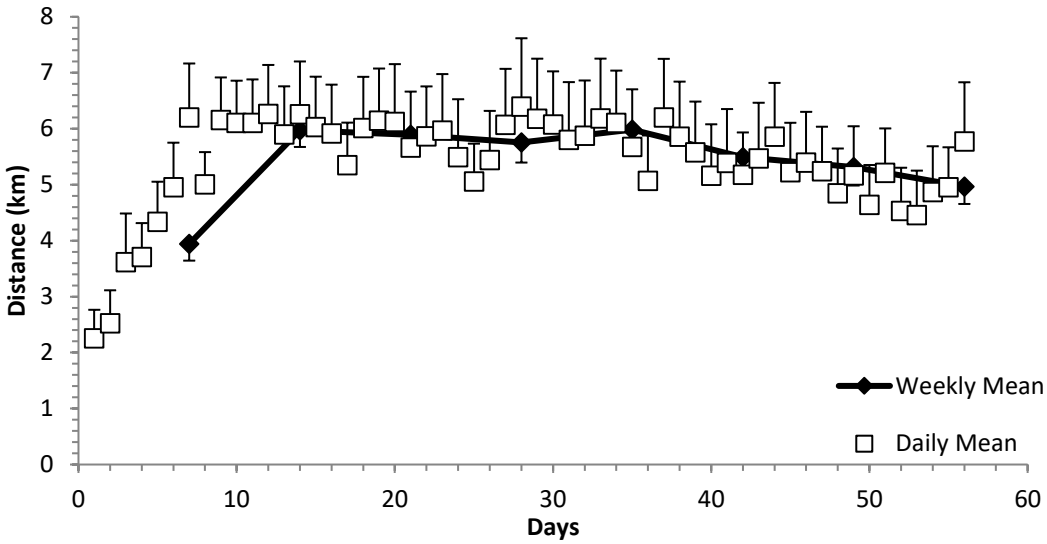
Distance (km)
Days
Weekly Mean
Daily Mean
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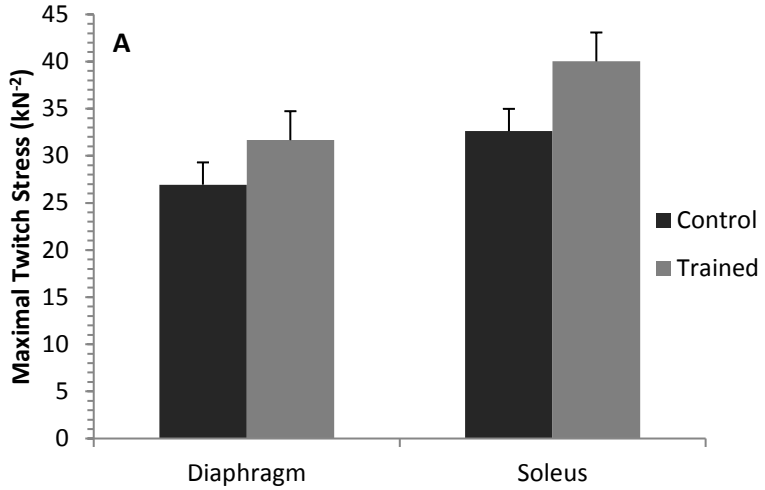
1
2
3
4
5
6
7
8
0
10
20
30
40
50
60

Figure 1


A
Maximal Twitch Stress (kN-2)
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20
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10
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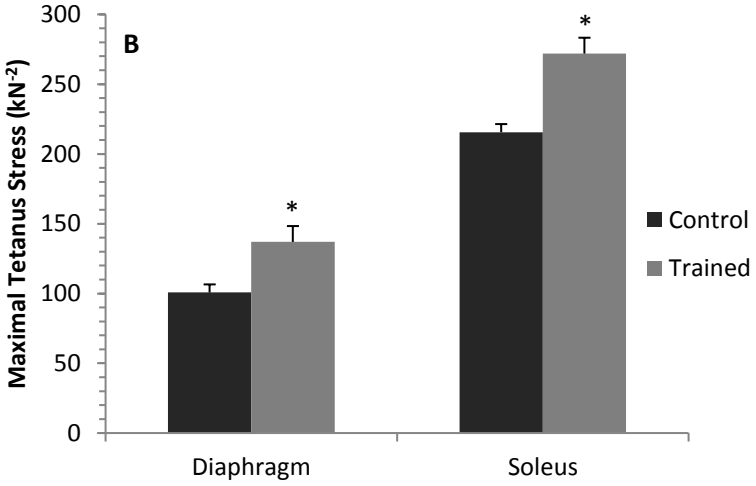
Diaphragm
Soleus
Control
Trained


B
Maximal Tetanus Stress (kN-2)
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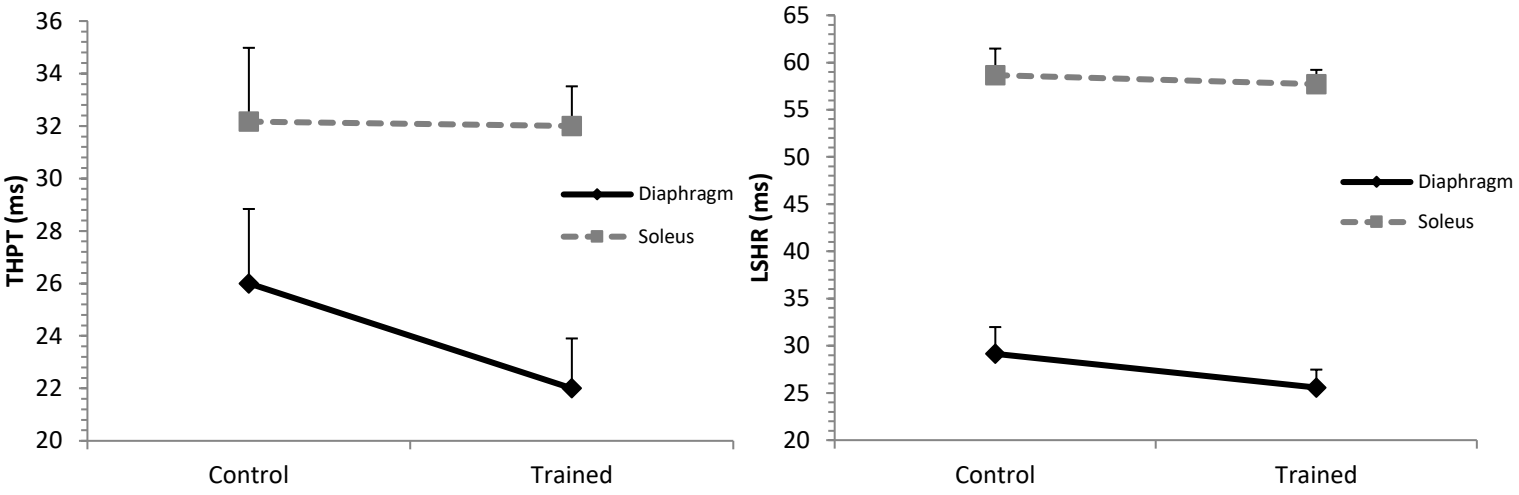
100
50
0
*
*
Diaphragm
Soleus
Control
Trained

Figure 2


THPT (ms)
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34
32
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Control
Trained
Diaphragm
Soleus
LSHR (ms)
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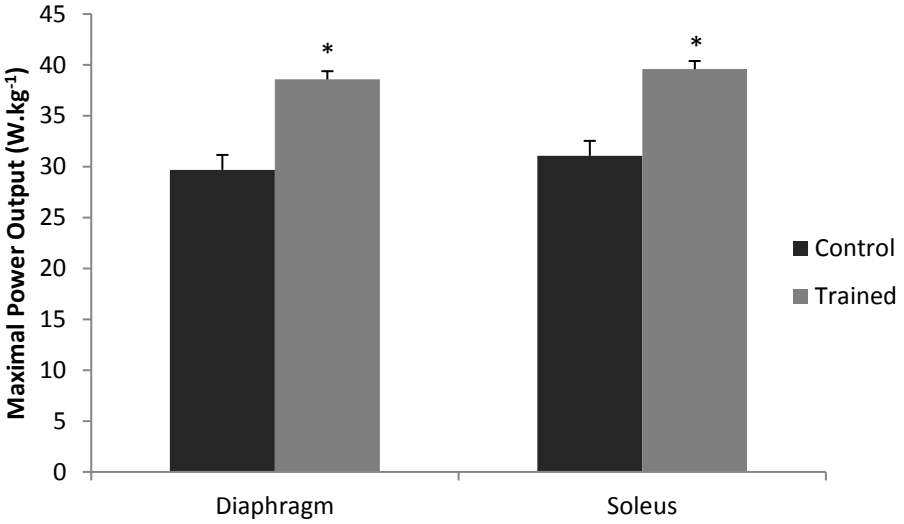
20
Control
Trained
Diaphragm
Soleus

Figure 3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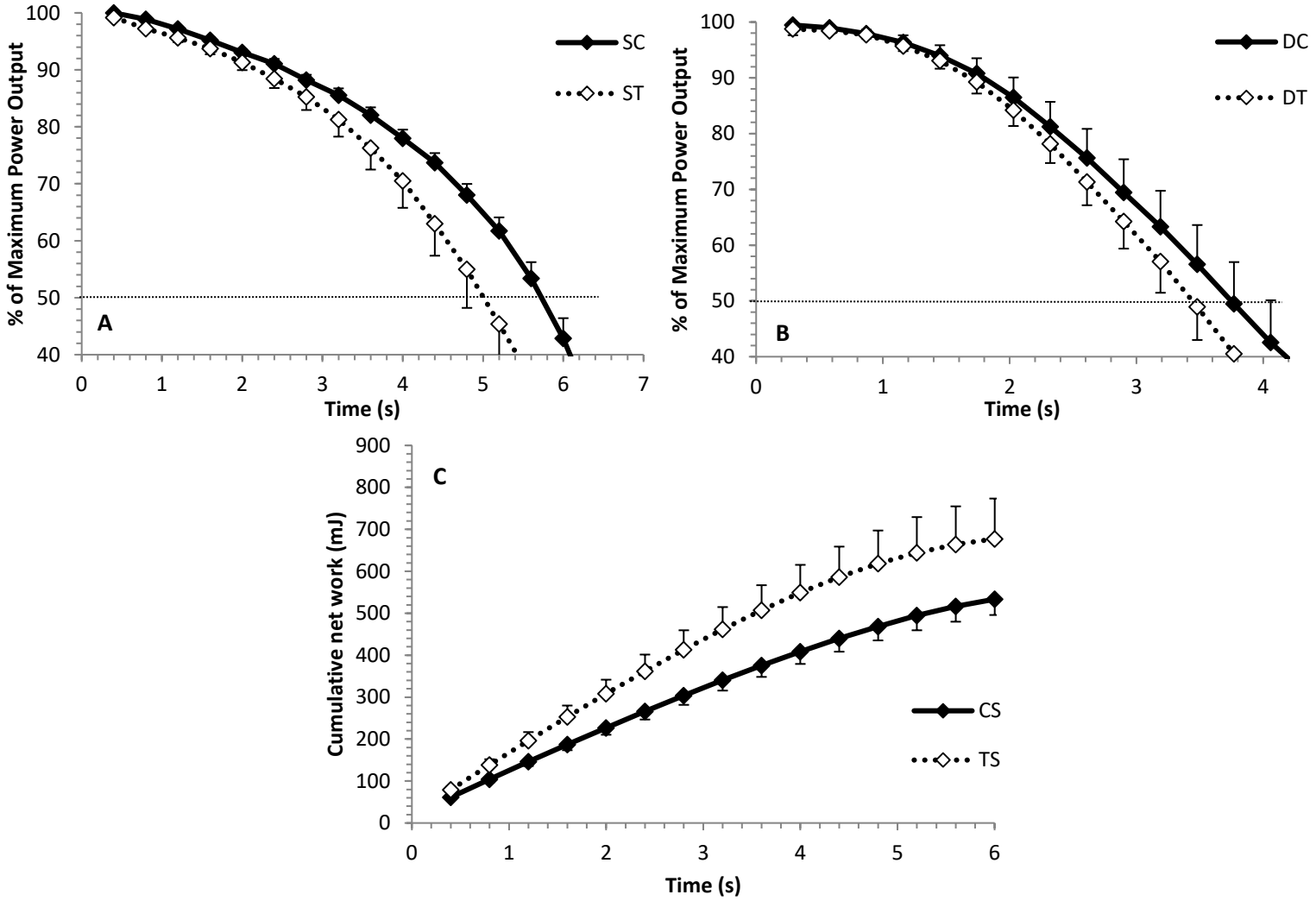
Maximal Power Output (W.kg-1)
*
*
Control
Trained
Diaphragm
Soleus

Figure 4


% of Maximum Power Output
Time (s)
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Cumulative net work (mJ)
CS
TS
C

Figure 5

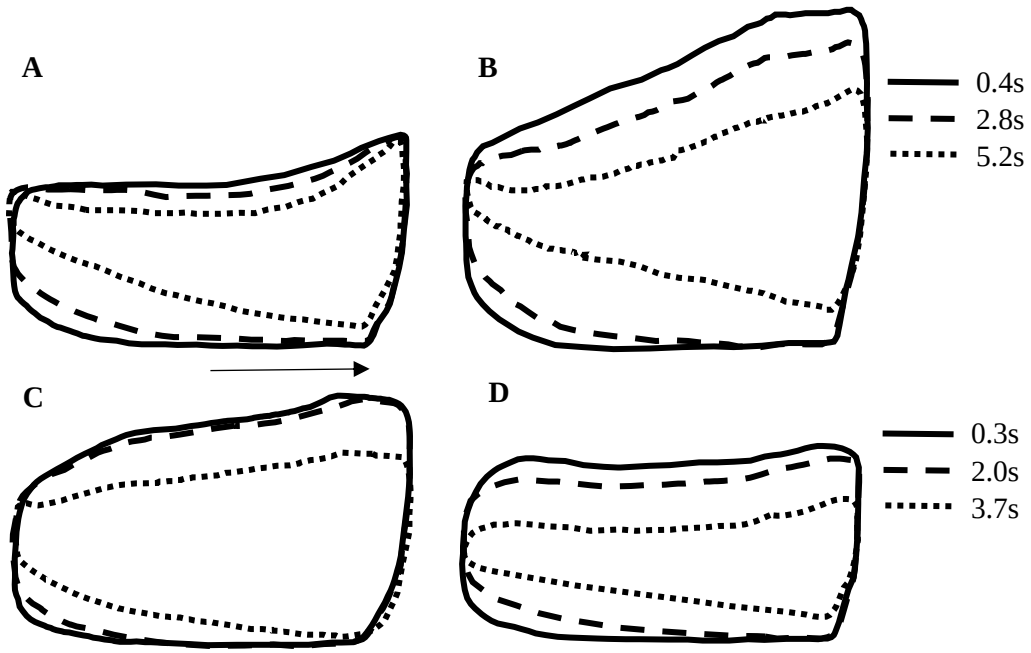

Figure 6.