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RESEARCH ARTICLE

Effect of stimulation frequency on force, power and fatigue of isolated mouse extensor digitorum longus muscle

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ABSTRACT

This study examined the effect of stimulation frequency (140, 200, 230 and 260 Hz) on isometric force, work loop (WL) power and the fatigue resistance of extensor digitorum longus (EDL) muscle (n=32), isolated from 8- to 10-week-old CD-1 female mice. Stimulation frequency had significant effects on isometric properties of isolated mouse EDL, whereby increasing stimulation frequency evoked increased isometric force, quicker activation and prolonged relaxation (P<0.047) up to 230 Hz and above; thereafter, force and activation did not differ (P>0.137). Increasing stimulation frequency increased maximal WL power output (P<0.001; 140 Hz, 71.3±3.5; 200 Hz, 105.4±4.1; 230 Hz, 115.5±4.1; 260 Hz, 121.1±4.1 W kg^{-1}), but resulted in significantly quicker rates of fatigue during consecutive WLs (P<0.004). WL shapes indicate impaired muscle relaxation at the end of shortening and subsequent increased negative work appeared to contribute to fatigue at 230 and 260 Hz, but not at lower stimulation frequencies. Cumulative work was unaffected by stimulation frequency, except at the start of the fatigue protocol, where 230 and 260 Hz produced more work than 140 Hz (P<0.039). We demonstrate that stimulation frequency affects force, power and fatigue, but these effects are not uniform between different assessments of contractile performance. Therefore, future work examining the contractile properties of isolated skeletal muscle should consider increasing the stimulation frequency beyond that needed for maximal force when examining maximal power but should utilise a sub-maximal stimulation frequency for fatigue assessments to avoid a high degree of negative work atypical of *in vivo* function.

KEY WORDS: Force, Power output, Fatigue, Work loop, Stimulation frequency, Muscle function

INTRODUCTION

The assessment of isolated skeletal muscle function has been integral to the current understanding of skeletal muscle mechanics and necessary in developing our understanding of the ageing process (Brooks and Faulkner, 1988; Brown and Hasser, 1996; Tallis et al., 2014), metabolic diseases (Tallis et al., 2017; Hurst et al., 2019; Eshima et al., 2017, 2020), nutrition (James et al., 2004; Tallis et al., 2012), and species diversity and evolution (Altringham and Johnston, 1986; Padilla et al., 2020; Stoehr et al., 2020). Except for measures of fatigue, the methodological approaches for the small number of techniques employed to assess the contractility of isolated skeletal muscle are typically standardised (Askew et al., 1997; James et al., 1995; Park et al., 2012; Moorwood et al., 2013). When utilising the work loop (WL) technique, which is an increasingly common assessment to measure power owing to its applicability to *in vivo* muscle mechanics (James et al., 1996; Nishikawa et al., 2018), the stimulation frequency that evokes peak isometric force is typically utilised (Ahn et al., 2003; James et al., 2004; Choi and Widrick, 2009; Tallis et al., 2017; Padilla et al., 2020; Hessel et al., 2021). However, this may limit our understanding of true physiological contractile performance as the stimulation frequency–force relationship for isometric conditions cannot reliably predict force output during dynamic contractions (Caiozzo, 2002; de Haan, 1998). Therefore, further research is needed to establish the optimal stimulation frequency for peak force and power, and to evoke a fatigue response that is more representative of *in vivo* fatigue skeletal muscle mechanics, which will ultimately improve the quality of data obtained.

The benefits of *in vitro* assessments of isolated skeletal muscle function have previously been discussed in detail (Josephson, 1993; Caiozzo, 2002; Syme, 2005; Nishikawa et al., 2018; Tallis et al., 2018, 2021). In brief, *in vitro* methodological approaches remove the confounding influence of the neural system, and so allow for direct assessments of muscle performance (Askew et al., 1997; James et al., 2004). The most commonly employed contractile assessment is the measurement of isometric force (Fulton, 1925; James et al., 1996; Medler, 2002; Syme, 2005; Nishikawa et al., 2018), which provides information on absolute force and stress (force normalised to cross sectional area), and through measuring the rate of activation and relaxation, provides insight into muscle calcium kinetics (Ebashi and Endo, 1968). Whilst isometric muscle activity has important *in vivo* applications, particularly for postural control (Loram et al., 2004), it fails to represent dynamic power-producing muscle activity (i.e. work done/time) that is essential for locomotion (Josephson, 1985; James et al., 1996).

Traditionally, assessments of isolated muscle power have been derived from force–velocity experiments, measured via isotonic or isovelocity assessments, which utilise a constant force or fixed shortening velocity, respectively (Josephson, 1993; Caiozzo, 2002). However, isotonic assessments overestimate power production, and have been shown to produce about twice the power output achieved via the WL method (James et al., 1996). The WL technique (Josephson, 1985) assesses the ability of a muscle to produce work during cyclical length changes, as per *in vivo* power-producing muscle (James et al., 1996). More specifically, power is derived from the net work of a WL length change cycle multiplied by the number of length change cycles per second (referred to as the cycle frequency) (James et al., 1996). As such, the WL technique considers not only the work produced during active muscle shortening but also work required to lengthen the muscle and the influence of activation and relaxation time (Josephson, 1985; James et al., 1996). One limitation of current WL protocols is that standard practice is to apply the stimulation frequency used to elicit maximal

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isometric tetanus force to the assessment of power (Ahn et al., 2003; Choi and Widrick, 2009; Hessel and Nishikawa, 2017; Hill et al., 2018; Padilla et al., 2020). This approach may be limited, given that the stimulation frequency–force relationship for isometric activity may not be applicable to dynamic conditions (de Haan, 1998; Caiozzo, 2002). In slow twitch soleus muscle, the stimulation frequency needed to evoke maximal power exceeds that needed for maximal isometric force (Vassilakos et al., 2009). However, this relationship may not be directly applicable to fast twitch muscle, such as the extensor digitorum longus (EDL). The EDL is composed of high levels of type IIx-IIb fibres (Brooks and Faulkner, 1991; Bobinac et al., 2000) and as such, the stimulation frequency required to achieve maximal isometric force is greater than in soleus (Tallis et al., 2013; Hill et al., 2018; Hurst et al., 2019) because of the need to evoke greater and sustained sarcoplasmic reticulum calcium release (Baylor and Hollingworth, 2003). Thus, the optimal stimulation frequency to elicit maximal WL power in type II fibres remains unknown.

Isolated muscle models are also used to assess fatigue resistance, typically utilising repeated isometric or WL activations (Askew et al., 1997; Syme and Tonks, 2004; Russ and Lovering, 2006; Choi and Widrick, 2009; Vassilakos et al., 2009; Kissane et al., 2018; Padilla et al., 2020). The ability to sustain force or power during repetitive muscular contractions has important *in vivo* applications, particularly for effective locomotion and health (Karatzaferi and Chase, 2013). Whilst the parameters used in fatigue protocols are more wide ranging than assessments of muscular force and power, and are often not well justified or lack *in vivo* relevance, generally, the stimulation parameters used to evoke peak force and power are used to elicit fatigue (Syme and Tonks, 2004; Tallis et al., 2013; Hill et al., 2018; Kissane et al., 2018). However, in some instances, this approach can result in a high degree of activation during re-lengthening and a subsequent increase in negative work (Tallis et al., 2013; Hill et al., 2018; Kissane et al., 2018), atypical of *in vivo* fatigue mechanics, where fibre stimulation and length change waveforms can be manipulated from one length change to the next in order to maximise work and negate potentially damaging excessive negative work (Wakeling and Rozitis, 2005). In an attempt to counteract this, some studies have made arbitrary adjustments in stimulation frequency, burst duration and cycle frequency (James et al., 2011; Seebacher et al., 2014), where the impact of these manipulations have not been systematically considered. Given that stimulation frequency influences the magnitude of calcium in the muscle cytoplasm, a reduction in stimulation frequency may play an important role in negating excessive negative work and may evoke equal or greater cumulative work during the WL assessment of fatigue, though this has yet to be systematically explored in fast twitch muscle.

Current evidence suggests that the standardised practice of utilising fixed stimulation parameters for all contractile assessments may underestimate true WL power output and produce a pattern of fatigue atypical of an *in vivo* response. As such, the present work aimed to determine the effects of stimulation frequency on maximal isometric force, activation and relaxation kinetics, WL power output and fatigue resistance of whole isolated fast twitch mouse EDL muscle. Such work provides the first comprehensive insight into the effect of stimulation frequency on fast twitch muscle mechanics, important for furthering the understanding of muscle physiology and for the future utility of isolated skeletal muscle assessments. We hypothesise that the stimulation frequency needed to evoke maximal power output will exceed that needed to for maximal isometric force and that higher stimulation frequencies during fatiguing contractions will result in prolonged relaxation and greater negative work, a faster reduction in peak power and reduced cumulative work.

MATERIALS AND METHODS

The procedures outlined in this study and the use of animals was approved by the ethics committee of Coventry University (P108131). Female CD-1 mice (Charles River, Kent, UK) aged between 8 and 10 weeks (body mass, 29.6±0.7 g. N=19) were used in the experimental procedures.

Experimental setup

Animals were culled via cervical dislocation (in accordance with the British Home Office Animals Scientific Procedures Act 1986, Schedule 1). Whole extensor digitorum longus (EDL) was rapidly isolated from either one (N=6) or both hindlimbs (N=13), resulting in a total sample N=32. An *a priori* power analysis (conducted in G*power v.3.1.9.7; power: 0.8, alpha: 0.05, effect size: 0.25) for a repeated measures ANOVA, indicated that a total sample of N=24 would be appropriate for the current study. EDL were isolated in refrigerated (1–3°C) oxygenated (95% O_2:5% CO_2) Krebs Henseleit solution (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 in each case; pH 7.55 at room temperature). For each EDL, the tendon and proximal bone were left intact and an aluminium foil T-clip was wrapped around the distal tendon, as close to the muscle as possible to avoid slippage when the muscle was producing force (Ford et al., 1977; Goldman and Simmons, 1984; James et al., 1995; Tallis et al., 2012). Each EDL muscle (N=32) was placed in a Perspex flow-through chamber filled with circulating oxygenated Krebs Henseleit solution. The temperature within the bath was continuously monitored using a digital thermometer (Traceable, Fisherbrand, Fisher Scientific, Loughborough, UK) and adjusted accordingly to maintain a physiologically relevant 37°C (±0.2°C range) via an external heater/cooler (Grant LTD6G, Grant Instruments, Shepreth, UK). Using the bone at the proximal end, the muscle was attached to a crocodile clip connected to a force transducer (UF1, Pioden Controls Ltd, Henwood Ashford, UK) and the T-foil clip at the distal end was attached to a crocodile clip, connected to a motor arm (V201, Ling Dynamic Systems, Royston, UK). The muscle was electrically stimulated to produce force via parallel platinum electrodes submerged in the Krebs solution inside the muscle chamber. The electrical currents were provided by a tabletop power supply (PL320 Thurlby Instruments, Huntington, UK). Initially, muscle length and stimulation amplitude (typically 14–18 V) were optimised to produce maximal isometric twitch force, as determined via digital storage oscilloscope (2211 or 1002, Tektronix, Marlow, UK). The muscle optimal length for isometric twitch performance was measured, using an eyepiece graticule and microscope, and defined as L_0. Estimated fibre length for the EDL (8.2±0.3 mm) was calculated as 75% of L_0 (James et al., 1995). During isometric and WL protocols, stimulation and length change parameters were controlled using custom-written software (Testpoint, CEC) via a D/A board (KPCI3108, Keithley Instruments) on a standard desktop personal computer or laptop.

Contractility measures

Isometric force

The procedures utilised to measure the contractile properties of isolated mouse EDL in this study are based on well-established protocols that have been utilised within the field for several decades, many of which have been adopted in work published from our

laboratory. Using the established optimal parameters for isometric twitch performance, the muscle was then subjected to a train of electrical stimuli (250 ms) across several stimulation frequencies (140, 200, 230 and 260 Hz). With the exception of 200 Hz, which was implemented first in order to be used as the control stimulation frequency in all muscles, isometric tetanus force was recorded across the range of stimulation frequencies in a randomised order. A recovery period of 5 min was implemented between each tetanus (Askew and Marsh, 1997; James et al., 1996). Once a tetanus had been performed at each experimental stimulation frequency, a control tetanus (200 Hz) was performed to monitor change in performance over time. Time to half peak tetanus (THPT) and last stimulus to half tetanus relaxation time (LSHR) were measured at each stimulation frequency as indicative measures of activation and relaxation time, respectively (Ebashi and Endo, 1968).

Work loop power output

Following measurements of isometric performance, power output was measured using the WL technique (Machin and Pringle, 1959; Josephson, 1985, 1993). The WL technique involved each muscle being subjected to four sinusoidal length changes around L_0 and stimulated to produce force during shortening. Length changes were driven via a motor (V201, Ling 220 Dynamic Systems, Royston, UK), the position of which was measured using a Linear Variable Displacement Transformer (DFG5.0, Solartron Metrology, Bognor Regis, UK). During the length change cycle, instantaneous force and velocity were sampled at 10 kHz and plotted against each other, forming a WL. Instantaneous power output was calculated for every data point in each WL cycle by multiplying instantaneous velocity by instantaneous force. Instantaneous power output values were averaged to generate an average power output for each length change cycle (Van Wassenbergh et al., 2007; Vanhooydonck et al., 2014).

The previously determined L_0 and stimulation amplitude which evoked maximal isometric force were implemented for the WL assessment of power output, as has been utilised in previous research examining WL power output of the EDL (James et al., 1995). The following parameters were standardised and utilised for each muscle preparation, based on previous work indicating that these parameters elicit maximal WL power output of the EDL of young female mice of a similar or same strain (James et al., 1995; Tallis et al., 2014, 2017; Hill et al., 2018). The cycle frequency of length change was set at 10 Hz. A strain of 0.10 of L_0 was implemented, resulting in the muscle lengthening by 5%, shortening by 10% before being re-lengthened by 5% back to L_0. Burst duration, 50 ms, was used to elicit maximal net work during shortening. The phase, i.e. time stimulation starts prior to reaching maximal length, was set at −2 ms as activation is not instantaneous, thus ensuring the muscle is active upon shortening. After each set of four WL cycles, a 5 min rest period was implemented to allow for sufficient recovery (Altringham and Young, 1991; James et al., 1995; Young and Rome, 2001). To examine the effect of stimulation frequency on WL power output, a range of stimulation frequencies (140, 200, 230 and 260 Hz) were used. The order in which stimulation frequencies were performed was randomised, except for 200 Hz, which was used as the control stimulation frequency in all trials to measure change in performance over time. Utilising a control stimulation frequency allows for the correction of WL power, which declines slowly over time owing to the build-up of a small anoxic core (Barclay, 2005). A linear decline in the contractile function of isolated mouse skeletal muscle over time has been previously demonstrated (James et al., 1995; Tallis, 2013) and to account for this, all WL power outputs were corrected assuming a linear decline in performance as per previous work (Vassilakos et al., 2009; Hurst et al., 2019; Hill et al., 2020).

Fatigue resistance

The effect of stimulation frequency (140, 200, 230 and 260 Hz) on fatigue resistance was measured by subjecting the EDL to 50 consecutive WL cycles, using the same parameters implemented for assessment of maximal WL power output (cycle frequency, 10 Hz; strain, 0.10; burst duration, 50 ms; phase, −2 ms). Only one fatigue run was performed per muscle (n=8 for each stimulation frequency). The change in WL power output was plotted as percentage decline relative to maximum power produced during the fatigue run (Tallis et al., 2014; Hurst et al., 2019; Hill et al., 2020). Assessing the relative decline in WL power output over the time course of the fatiguing contractions provides information regarding the impact of stimulation frequency on the rate of fatigue. However, cumulative work, calculated as the sum of the net work performed in each cycle (Askew et al., 1997) was also determined to infer absolute differences in the fatigue response, accounting for higher work initially expected at greater stimulation frequencies but associated with a faster rate of fatigue. WL shapes were plotted as force against strain (%L_0) over 2500 individual force and length data points for each work loop cycle. The components of a typical WL cycle of mouse EDL optimised for maximal work at 10 Hz cycle frequency using a 260 Hz stimulation frequency are provided in Fig. 1 as an example of how a work loop cycle is plotted. Following the fatigue protocol, the ability of each muscle to recover concentric power production was measured every 10 min for 30 min, using a fixed stimulation frequency of 200 Hz. The recovery of power was expressed as a percentage relative to the pre-fatigue control WL power output.

Muscle size calculations

Upon completion of contractile assessments, the muscle was detached from the crocodile clips and removed from the muscle chamber. The tendons, T-foil clip and bone were removed, leaving only muscle tissue, which was then blotted on absorbent paper to remove the excess Krebs solution and weighed to determine wet muscle mass (muscle mass=8.9±0.3 mg; TL-64, Denver Instrument Company, Arvada, CO). Mean muscle cross-sectional area was calculated from L_0, muscle mass, and an assumed density of 1060 kg m^{-3} (Méndez and Keys, 1960). Maximal isometric stress (kN m^{-2}) at each stimulation frequency was calculated by dividing peak tetanic force by mean muscle cross sectional area (CSA). Absolute power output (watts) was calculated as net work (work during shortening minus work during lengthening) multiplied by cycle frequency. WL power output normalised to muscle mass (W kg^{-1} muscle mass) was calculated by dividing absolute power output by muscle mass.

Statistical analysis

Statistical analysis was performed using R Studio (R Foundation for Statistical Computing, Version 4.0.4; https://www.r-project.org/). All data were normally distributed (checked via histogram using descriptive statistics) with acceptable limits of skewedness of ≤±2 observed (Gravetter and Wallnau, 2014) and showed homogeneity of variance (checked via Levene's test) thus, parametric analysis was performed. A repeated measures mixed effects model, using the lmer test (https://CRAN.R-project.org/package=lmerTest) package in RStudio, assessed the effect of stimulation frequency on isometric stress, THPT, LSHR and WL power output normalised to muscle mass. The random effect of specimen was used as both EDL (n=13) were isolated from most individuals (Hurlbert, 1984).

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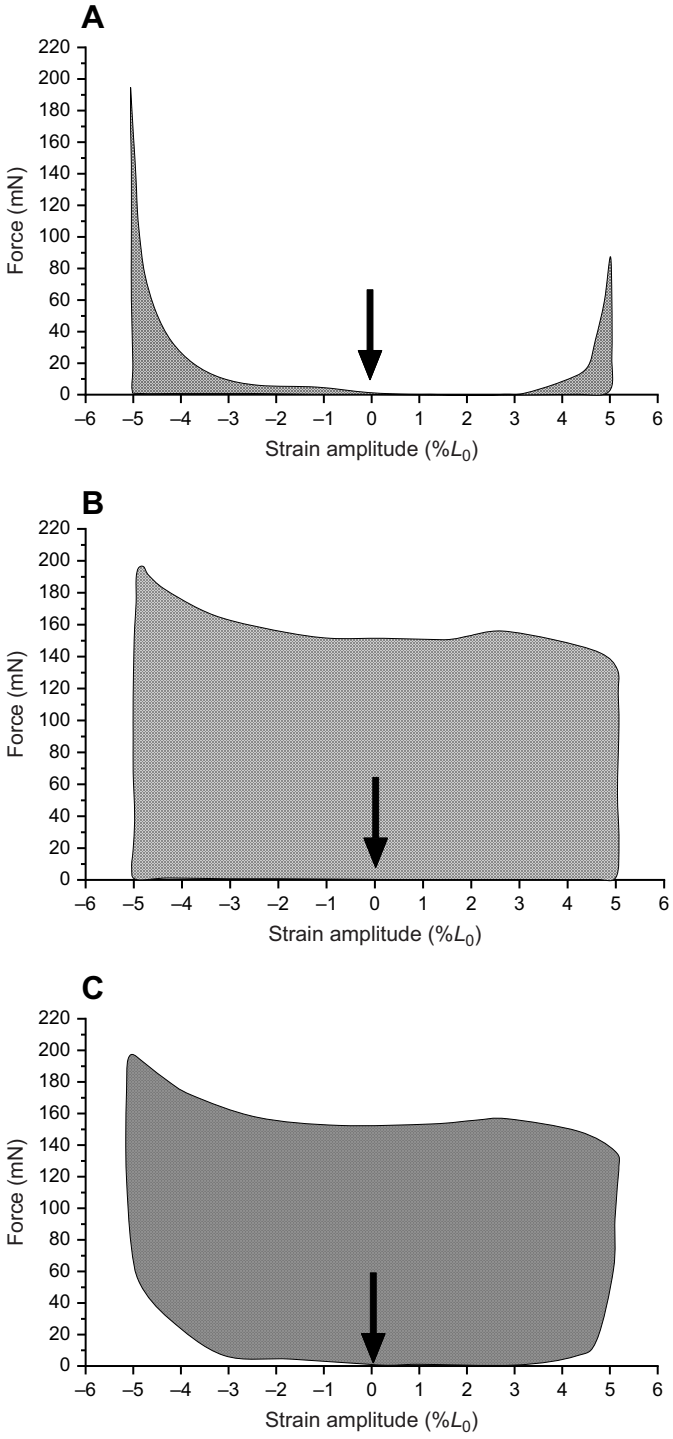

Fig. 1. Components of a mouse extensor digitorum longus work loop cycle optimised for maximal work at 10 Hz cycle frequency and 260 Hz stimulation frequency. (A) Work performed on the muscle during lengthening (negative work). (B) Work performed by the muscle during the shortening phase of the length change cycle. (C) Net work produced during the entirety of the length change cycle, calculated as total work minus negative work (C=B–A). Work loops are performed in the anti-clockwise direction, with the initiation of the work loop starting at L_0 as indicated via the arrow.

We used a mixed effects model, with stimulation frequency and time as factors, to assess effect of the stimulation frequency used during the fatigue protocol on the ability of the EDL to recover maximal WL power output. The random effect of specimen was allowed to vary using random intercepts and slopes. Significant main effects and interactions observed were explored using Tukey's pairwise comparisons. Cohen's *d* was calculated to measure effect size and was then corrected for bias using Hedge's *g* according to the appropriate sample size (Hedges, 1981). Cohen's *d* effect size was interpreted as trivial (<0.2), small (0.2–0.6), moderate (0.6–1.2) or large (>1.2) (Hopkins et al., 2009).

Using the SPM–1D package (Todd Pataky, v. M 0.1) in MATLAB (The MathWorks Inc, R2018b, Natick, MA, United States), we performed statistical parametric mapping (SPM) on cumulative work and percentage decline in power output data obtained during the fatigue protocol (Pataky, 2010). SPM calculates the *F* (ANOVA) or *t* (*t*-test) value on every data point obtained during the fatigue protocol, but instead of calculating a *P* value for every data point, inferential statistics are based on random field theory and thus maintain a constant error of α (Pataky et al., 2013). We first performed one-way analysis of variance (ANOVA) SPM[F] statistics to determine if there was a main effect of stimulation frequency on cumulative work and percentage decline in fatigue relative to maximum power output. A *P* value was calculated where clusters crossed the critical threshold (Pataky et al., 2013). Where main effects were observed, *post hoc* 2-sample SPM[t] (two-sided *t*-test) were conducted between each stimulation frequency separately to identify which stimulation frequencies differed and where the differences occurred (Pataky et al., 2015). Where clusters crossed the critical threshold, this indicated a significant difference at $P \leq 0.05$. All data are presented as mean±s.e.m. All data were normally distributed, with the level of significance was set at $P \leq 0.05$.

RESULTS

Isometric tetanic stress

The mixed model repeated measures identified a significant effect of stimulation frequency on maximal isometric tetanic stress (Fig. 2A; $P<0.001$; 140 Hz, 244.5±9.3; 200 Hz, 293.1±8.5; 230 Hz, 315±10.9; 260 Hz, 326.3±8.8 kN m^{-2}). Tukey's multiple comparisons indicated significant differences in maximal isometric tetanic stress between most stimulation frequencies (140 versus 200 Hz: $P<0.001$, $d=0.96$; 140 versus 230 Hz and 260 Hz: $P<0.001$, $d>1.23$; 200 versus 230 Hz: $P=0.008$, $d=0.4$; 200 versus 260 Hz: $P<0.001$, $d=0.68$) except between 230 Hz and 260 Hz ($P=0.237$, $d=0.19$).

The mixed model repeated measures identified a significant effect of stimulation frequency on time to half peak tetanus (THPT) (Fig. 2B. $P<0.001$; 140 Hz, 18.4±0.9; 200 Hz, 15.2±0.9; 230 Hz, 13.0±0.7; 260 Hz, 12.5±0.7 ms). Tukey multiple comparisons indicated significant differences in THPT (140 versus 200 Hz: $P<0.001$, $d=0.81$; 140 versus 230 Hz and 260 Hz: $P<0.001$, $d>1.54$; 200 versus 230 Hz and 260 Hz: $P<0.002$, $d>0.63$) between all stimulation frequencies except between 230 and 260 Hz (THPT: $P=0.687$, $d=0.15$). Furthermore, we identified a significant effect of stimulation frequency on last stimulus to half relaxation time (Fig. 2C; $P<0.001$; 140 Hz, 7.6±0.3 ms; 200 Hz, 8.8±0.3 ms; 230 Hz, 12.1±0.5 ms; 260 Hz, 13.4±0.5 ms). Tukey multiple comparisons indicated significant differences in LSHR between all stimulation frequencies, with relaxation time increasing with stimulation frequency LSHR (140 versus 200 Hz: $P<0.001$, $d=1.01$; 140 versus 230 Hz and 260 Hz: $P<0.001$, $d>2.39$; 200 versus 230 Hz and 260 Hz: $P<0.001$, $d>1.79$; 230 versus 260 Hz: $P=0.004$, $d=0.59$).

Work loop power output

The mixed model repeated measures identified a significant effect of stimulation frequency on WL power output normalised to muscle

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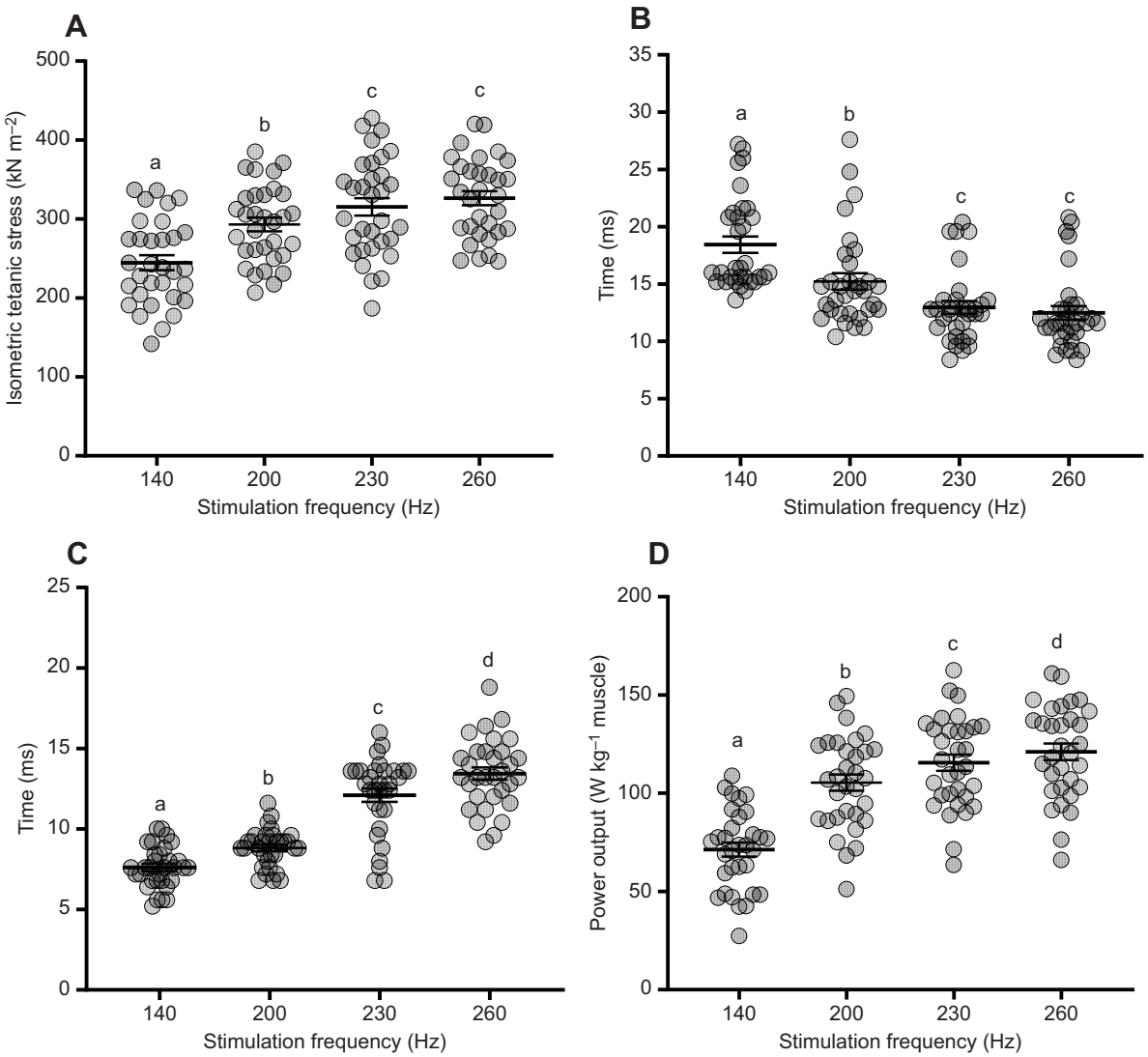

Fig. 2. Effect of stimulation frequency on contractile performance of mouse EDL muscle. (A) Maximal isometric tetanic stress, (B) time to half peak tetanus, (C) last stimulus to half tetanus relaxation time and (D) maximal work loop power output of EDL at 4 stimulation frequencies. Data are presented as mean±s.e.m. muscle force values normalised to muscle cross sectional area, muscle power normalised to muscle mass; *N*=32. Data with different letters (a,b,c.d) are significantly different at $P<0.05$, determined via mixed model repeated measures ANOVA.

mass (Fig. 2D; $P<0.001$; 140 Hz, 71.3±3.5; 200 Hz, 105.4±4.1; 230 Hz, 115.5±4.1; 260 Hz, 121.1±4.1 W kg^{-1}). Tukey multiple comparisons indicated significant differences in WL power output between all stimulation frequencies, with higher stimulation frequency resulting in greater WL power output (140 versus 200, 230 Hz and 260 Hz: $P<0.001$, $d>1.58$; 200 versus 230 Hz: $P<0.001$, $d=0.44$: 200 versus 260 Hz: $P<0.001$, $d=0.68$; 230 versus 260 Hz: $P<0.001$, $d=0.24$).

The effects of stimulation frequency on fatigue resistance

Statistical parametric analysis (SPM) ANOVA[F] indicated a significant effect of stimulation frequency on percentage decline of power output relative to maximum ($P<0.05$). As summarised in Fig. 3A,B (see also Fig. S1), the SPM[*t*] *t*-test results indicated that higher stimulation frequencies fatigue quicker at various time points (Fig. 3B; 140 Hz versus 200 Hz: $P<0.001$ between WL 14–42 [1.86–5.04 s]; 140 Hz versus 230 Hz, $P<0.001$ between WL10 and WL38 [1.2–4.56 s]; 140 versus 260 Hz, $P<0.001$; between WL9 and WL40 [1.08–4.8 s]; 200 versus 230 Hz, $P<0.001$ between WL11 and WL28 [1.32-3.36 s]; 200 versus 260 Hz, $P<0.001$ between WL8 and WL32 [0.96–3.84 s]; 230 versus 260 Hz $P<0.001$ between WL11 and WL21 [1.32–2.52 s].

SPM ANOVA[F] also indicated a significant effect of stimulation frequency on cumulative work production ($P<0.05$). As summarised in Fig. 3C,D (see also Fig. S2), the SPM[*t*] *t*-test results indicated that 230 and 260 Hz initially produce greater work when compared to 140 Hz (Fig. 3D; 140 versus 230 Hz: $P=0.039$ between WL1 and WL4 [0.12–0.48 s]; 140 Hz versus 260 Hz: $P<0.001$ between WL1 and WL18 [0.12–2.16 s]), but other stimulation frequencies did not significantly differ at later time points ($P>0.05$).

The area within the work loop represents the net work done during the length change cycle. Typical work loop shapes, presented in Fig. 4, demonstrate that initial net work is larger at 230 and 260 Hz (Fig. 4C,D) when compared with 140 and 200 Hz (Fig. 4A,B). However, from WL10, there is greater force production during muscle re-lengthening for muscle stimulated at 230 and 260 Hz distorting the work loop shape, and reducing the net work,

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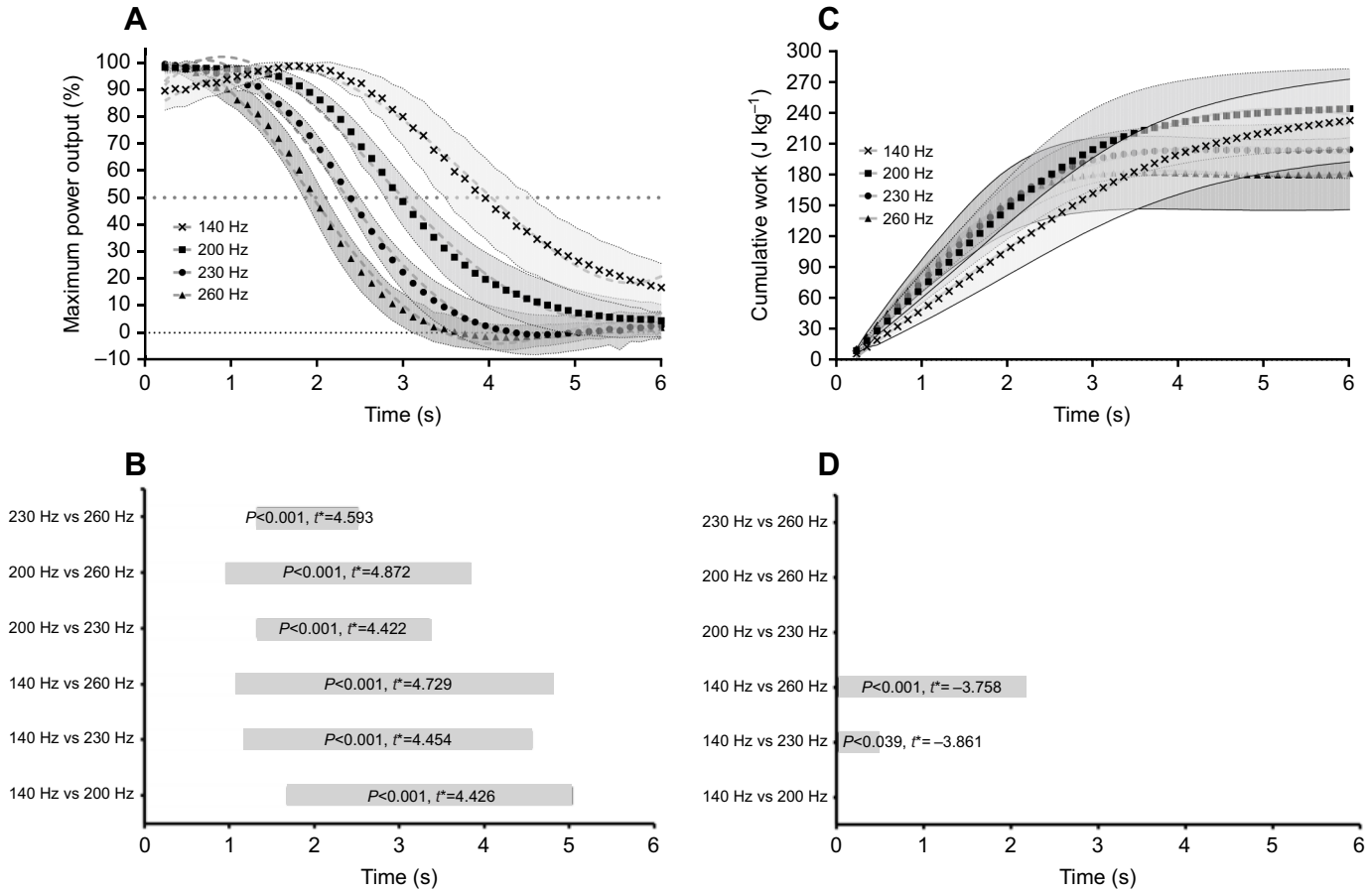

Fig. 3. Effect of stimulation frequency on net muscle power output, relative to maximum and cumulative work production, during fatigue at 10 Hz cycle frequency for mouse EDL at 37°C. (A) Net muscle power output relative to maximum. (C) Cumulative work production. Results from two-sample SPM[*t*] statistical analysis for net muscle power output relative to maximum (B) and cumulative work production (D) are described directly beneath their respective figure. Shaded part of figure indicates statistical difference between specific stimulation frequencies at $P<0.05$. Data presented as mean±95% confidence interval in A and C; $N=8$ at each stimulation frequency.

to a much greater degree than seen at the lower stimulation frequencies. As a result, from WL17 there is a marked reduction in the size of the WL shape relative to the initial WL at the higher stimulation frequencies, whereas, in contrast, WL shape is comparatively well maintained at the lower stimulation frequencies.

Recovery

For recovery of maximal power output, assessed every 10 min for 30 min post 50 consecutive WL cycles, there was no significant effect of stimulation frequency (Fig. 5; $P=0.216$; 140 Hz, 66.3±4.9; 200 Hz, 50.7±3.9; 230 Hz, 51.9±6.8; 260 Hz, 53.9±6.7% of control power output pre-fatigue after 30 min). There was a significant effect of time ($P<0.001$), indicating significant recovery every 10 min, irrespective of stimulation frequency. There was no significant stimulation frequency×time interaction ($P=0.060$).

DISCUSSION

The novel examination of the effects of stimulation frequency on the contractile performance of fast twitch mouse EDL muscle found that stimulation frequency has significant effects on force production, activation and relaxation rates, power output and pattern of fatigue, but these effects are contractile assessment specific. Furthermore, the current data indicate that a sub-maximal stimulation frequency should be considered for the assessment of fatigue resistance in order to minimise the contribution of negative work to the decline in fatigue and more accurately reflect the fatigue response that occurs *in vivo*.

Effect of stimulation frequency on isometric contractile mechanics

Whole isolated EDL muscles produced a maximal isometric tetanic stress of 326±9 kN m^{-2}, which is comparable to values of 300±23 and 332±9 in mice of the same strain, age and gender, and contractile performance measured at the same experimental temperature of 37°C (Tallis et al., 2012; Hill et al., 2018). Comparisons for maximal EDL stress can also be made across the scientific literature, where EDL stress has shown to range from 200 to 320 kN m^{-2} (James et al., 1995; Askew and Marsh, 1997; Hessel and Nishikawa, 2017; Debruin et al., 2019; Hayes et al., 2019; Eshima et al., 2020), although direct comparisons between these studies should be made with caution given the variation in temperature, age, gender and strain of mice, which are likely to influence tetanic stress.

Higher stimulation frequency resulted in greater isometric stress and quicker activation rates, until a threshold (230 Hz and above) beyond which a further increase in stimulation frequency had little effect. This supports previous work which indicates that lower stimulation frequencies result in a reduction in tetanic stress of mouse EDL (Tallis et al., 2012) and data that show surplus stimulation beyond the optimal has little effect on the isometric force-producing capacity of mammalian muscle (Buller and Lewis,

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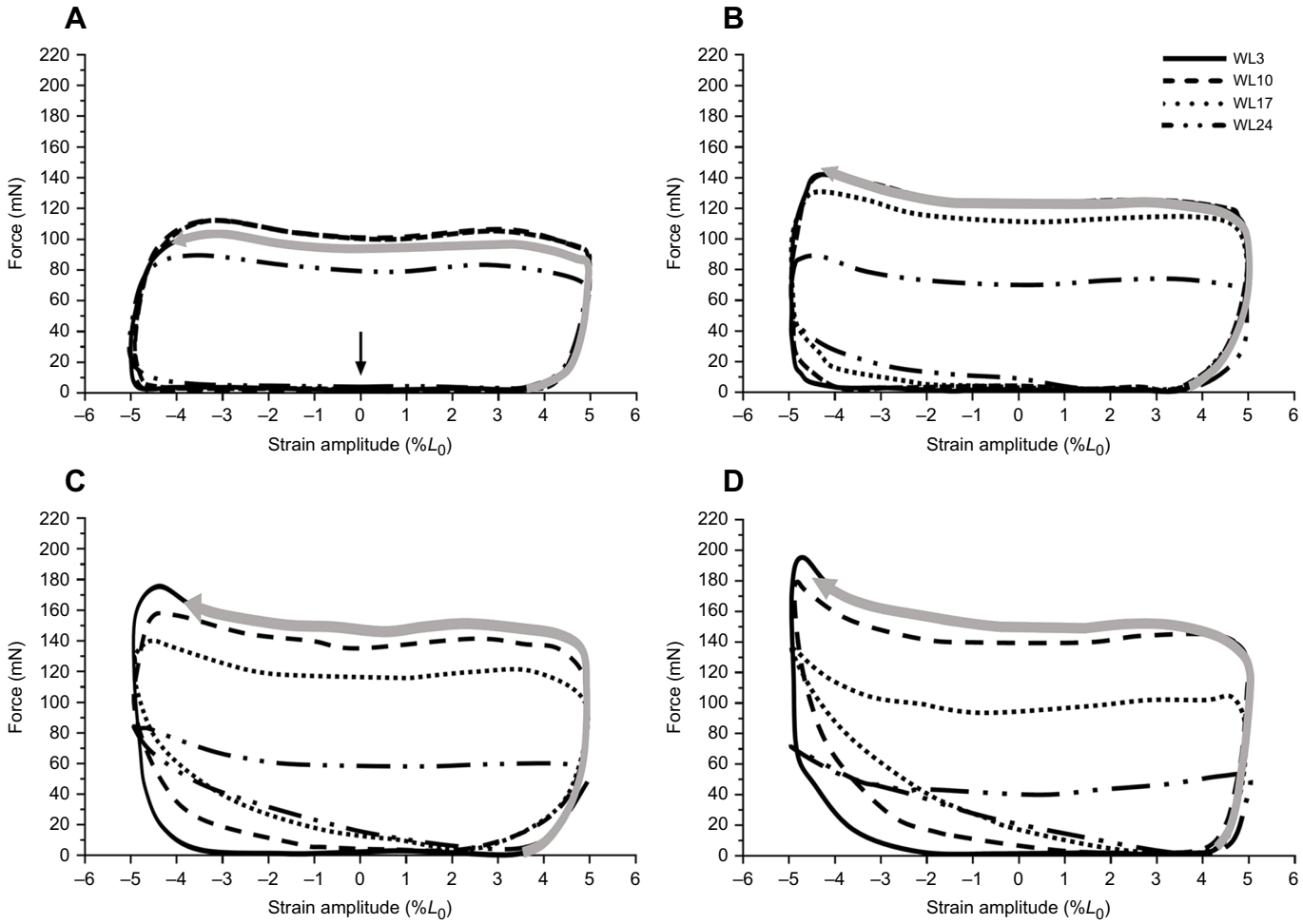

Fig. 4. Effect of stimulation frequency on typical work loop shapes during muscle fatigue at 10 Hz cycle frequency for mouse EDL. Figures are plotted as force against strain (%L_0) for stimulation at (A) 140 Hz, (B) 200 Hz, (C) 230 Hz and (D) 260 Hz. Work loop (WL) 3, 10, 17 and 24 of the fatigue protocols are shown for each group. Work loops are performed in the anti-clockwise direction, with the work loop starting at L_0, indicated via arrow in A. The stimulation duration (grey overlay) is shown for work loop 3 and is the same for all stimulation frequencies.

1965; de Haan, 1998; Vassilakos et al., 2009). Our findings also support the idea that isometric activation time increases with stimulation frequency, in line with previous literature (Buller and Lewis, 1965; de Haan, 1998; Vassilakos et al., 2009). However, force production and activation time appear to plateau at the same stimulation frequency, which differs from previous work in the soleus, where a stimulation frequency greater than that required to elicit peak force produced a shortened activation time (Vassilakos et al., 2009). The alignment of an optimised stimulation frequency for both peak force and activation time for EDL likely reflects a strategy to evoke effective rapid high force output, a requirement of fast twitch muscle (Radák, 2018).

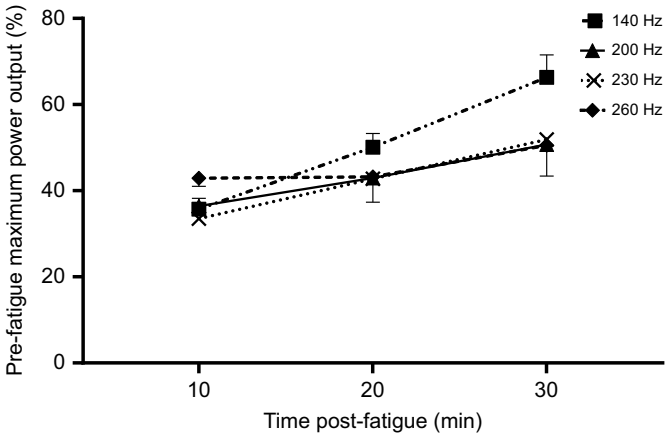

Fig. 5. Effect of stimulation frequency on recovery of maximal work loop power output of mouse EDL over 30 min post-fatigue. Data presented as means±s.e.m.; *N*=8 at each stimulation frequency; for clarity, not all s.e.m. bars are shown.

Effect of stimulation frequency on work loop power output

Our work, whilst in agreement with previous literature which indicates maximal WL power output is greater at higher stimulation frequencies (Vassilakos et al., 2009; Tallis et al., 2012, 2013), extends beyond what has previously been reported, as previous work which has considered stimulation effects on WL power output of mouse EDL did not explore stimulation frequencies beyond that used to elicit maximal isometric force. The present data identifies that whole isolated EDL muscles produced a maximal WL power output of 121 ±4 W kg^{-1} at a 260 Hz stimulation frequency, which is approximately 14–20% greater than values of 99±5 W kg^{-1} and 105±6 W kg^{-1} reported in previous work (Hill et al., 2018; Hurst et al., 2019). The greater power output per unit of tissue mass

observed is likely a result of optimising stimulation frequency for WL power output, which is evident given that the WL power output values from muscle subjected to 200 Hz stimulation frequency in this study (105±4 W kg^{-1}) are comparable to previous work (99±5 and 105±6 W kg^{-1}) that utilised a single fixed stimulation frequency of 200–220 Hz using the same strain of mice (Hill et al., 2018; Hurst et al., 2019). Based on our findings, it is plausible that previous research that has implemented the stimulation frequency for optimal isometric performance of mouse EDL may have underestimated true maximal WL power output. The greater simulation frequency needed to elicit peak power is unlikely to be attributable to an increased activation time, given that there was no difference in THPT between 230 and 260 Hz. Thus, the increased power likely reflects an increased ability to generate force during shortening, which maximises positive work. Whilst we are the first to show that the stimulation needed to elicit maximal WL power output of mouse EDL exceeds that needed for maximal isometric force, our findings support the suggestion that a stimulation frequency–force relationship for isometric conditions cannot reliably predict force output during dynamic contractions (James et al., 1996; de Haan, 1998; Caiozzo, 2002; Vassilakos et al., 2009). Based on our data, we suggest that future projects utilising the WL technique to achieve maximal power output in fast twitch muscle should consider utilising a stimulation frequency greater than that which evokes peak isometric force.

Effect of stimulation frequency on muscle fatigue

The ability of the EDL muscle preparation to recover from fatigue and the trend of recovery was comparable to that of previous work (Tallis et al., 2014; Hill et al., 2018), and did not significantly differ between stimulation frequencies, indicating that the decline in the ability of the muscle to produce power during consecutive WLs is a result of peripheral fatigue and that the contribution of any muscle damage to a decline in power output during fatigue was minimal (Askew et al., 1997; James et al., 2004).

Similarly to previous work using soleus muscle (Vassilakos et al., 2009), at the start of the fatigue protocol higher stimulation frequencies (230 and 260 Hz) produced greater cumulative work than the lowest stimulation frequency (140 Hz). This is unsurprising given that higher stimulation frequencies initially elicited greater WL power output. However, as the fatigue protocol progressed, the lower stimulation frequencies elicit comparable cumulative work, attributed to the relative maintenance of power production throughout the fatigue protocol at those lower stimulation frequencies. By the end of 50 consecutive WL cycles, stimulation frequency did not influence cumulative work. Rate of fatigue was significantly quicker as stimulation frequency was increased, which largely supports observations made on relatively slow twitch mouse soleus muscle (Vassilakos et al., 2009; Tallis et al., 2013). Therefore, despite the initially high WL power output observed at higher stimulation frequencies, the ability to sustain power is significantly diminished, whereas power production is relatively well maintained at the lower stimulation frequencies.

The differences in time to fatigue and limited differences in cumulative work can, in part, be explained by examining the WL shapes. Whilst it may be intuitive to assume that muscle stimulated to evoke maximal power will fatigue more quickly, our data suggest that current protocols are limited given the fatigue elicited is partly induced by the assessment itself and not entirely a physiological response. WL shapes indicate that as the fatigue protocol progresses, higher stimulation frequencies elicit greater force during re-lengthening, thus increasing the negative work produced during the length change cycle, which reduces net work, and influences both cumulative work production and rate of fatigue. This increase in negative work during the progression of the fatigue protocol is less apparent at lower stimulation frequencies. The increased mechanical energy required to re-lengthen the muscle is likely a result of higher quantities of intracellular calcium limiting the ability of the muscle to remain predominantly passive during lengthening (Westerblad and Allen, 1994). Furthermore, increased negative work may also be attributed to differences in the mechanical function of titin. Recent evidence indicates that calcium activated titin is increasingly stiffened thus requiring greater work for muscular elongation (Tahir et al., 2020; Hessel et al., 2021). Whilst the stimulation frequency used for assessment of maximal isometric force and WL power output is typically utilised for assessment of fatigue (Tallis et al., 2014, 2017; Hill et al., 2018, 2019, 2020; Hurst et al., 2019) and it is common to observe prolonged relaxation and increased negative work during fatigue protocols which employ a large number of WL cycles (Askew et al., 1997; James et al., 2004; Tallis et al., 2014; Hill et al., 2018), current approaches may have limited *in vivo* application. The large negative work component seen in fatigue at higher stimulation frequencies is atypical of *in vivo* muscle mechanics, where stimulation would be altered to minimise the potential for eccentric activity, that would induce damage, and to maximise net work per cycle (Wakeling and Rozitis, 2005). Whilst there is complexity in replicating this precisely in an *in vitro* model, our data infer that a submaximal stimulation frequency likely better replicates the *in vivo* fatigue response to dynamic contractions and should be considered in the future application of the WL technique. Whilst some previous studies have reduced stimulation frequency used when determining fatigue responses (Seebacher et al., 2014), the rationale for choice of stimulation frequency is unclear. In the present study, 200 Hz for the EDL appears near optimal, producing a high amount of cumulative work, with the relative change in work done during shortening (as indicated by the WL shapes) being similar to that at 260 and 230 Hz, but with minimal negative work.

Limitations and future direction

One limitation of the present study and many studies using the WL technique (Josephson, 1985, 1993), is the use of sinusoidal length change waveforms. Sinusoidal length change waveforms are commonly used for WL assessments (James et al., 2004; Choi and Widrick, 2009; Tallis et al., 2014; Hessel and Nishikawa, 2017; Stoehr et al., 2020) as they provide a close approximation of the dynamic *in vivo* cyclical muscle activities in some species, such as fish during steady swimming and steady insect flight (Josephson and Ellington, 1997; Ellerby et al., 2000). However, in other species, muscles *in vivo* can often undergo complex length changes during real-life locomotion (Dickinson et al., 2000) and estimates of the muscle length change used by mouse EDL during running suggest deviation from a sinusoidal length change (James et al., 1995). Furthermore, as the tendon at the proximal end of the EDL remained intact during contractile assessments, it is possible that length change during repetitive fatiguing contractions would gradually reduce because of tendon creep evoked by sustained mechanical stress (Wren et al., 2003). Another limitation of the present work is that WL power output continued to rise with stimulation frequency, therefore it may be anticipated that a further increase in stimulation frequency beyond the 260 Hz used in the present study could elicit a further increase in power output. However, despite the difference in WL power between 230 and 260 Hz reaching statistical significance, the effect size of differences observed in WL power output between 230 and 260 Hz

was small (d=0.24), with a mean difference of ∼4%. Thus, we believe the effects of a further increase in stimulation frequency are likely to be minimal. Furthermore, stimulation frequencies greater than 260 Hz should not be implemented for assessment of fatigue for the EDL given that there is substantial negative work due to prolonged relaxation at above 230 Hz thus limiting the physiological relevance of higher stimulation frequencies.

One area of future direction is to consider the magnitude of cellular muscle damage evoked by stimulation frequency during repeated maximal contractions during fatigue. Whilst stimulation frequency did not affect the ability of the muscle to recover maximal power output, differences in muscle damage may still exist at the cellular level given that previous work indicates that the magnitude of force production is related to magnitude of z-line disruption, identified via transmission electron microscopy imagery (Mackey et al., 2008). Whilst the present study has provided important insight needed to better optimise power output via the WL technique, there is still opportunity to further refine the method. One important area of future direction is to consider optimising muscle length for dynamic contractions. At present, the muscle length optimal for isometric performance is often implemented in the WL assessment of mammalian muscle (James et al., 2005; Hessel and Nishikawa, 2017; Kissane et al., 2018; Hill et al., 2020). Whilst previous work has considered the effect of starting length on WL power output of the soleus and EDL, reporting reduced power at lengths 10 and 20% greater and less than L_0 (James et al., 1995), this was only performed at a single cycle frequency. Therefore, future work should consider the effects of starting length across a range of cycle frequencies, adjusting muscle length in smaller increments, and systematically adjusting each contractile parameter to achieve maximal net work at each length.

Conclusion

In summary, electrical stimulation frequency had significant effects on isometric properties, WL power output and pattern of fatigue of isolated mouse EDL, although the optimal stimulation frequency for maximal performance and *in vivo* applicability is not entirely uniform across the different contractile assessments. Higher stimulation frequency resulted in greater isometric stress, quicker activation rates, and prolonged relaxation periods until a threshold where further increase in stimulation frequency had little effect, except for LSHR which continued to increase. One of the key findings from the present study is that WL power output continued to increase beyond the stimulation frequency that evoked maximal isometric force. Thus, the current approach of implementing the stimulation frequency for optimal isometric force, for the assessment of power may underestimate true maximal WL power output. With respect to *in vivo* applicability of the WL technique's assessment of fatigue, our findings suggest the need for a submaximal stimulation frequency to ensure the rate and pattern of fatigue is primarily a physiological response, with minimal contribution induced by the technique itself. Based on the present data, future work may wish to consider using an optimal stimulation frequency for each contractile assessment of whole isolated EDL muscle to improve the quality of the data obtained.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: S.P.S., R.S.J., J.T.; Methodology: S.P.S., J.T.; Formal analysis: S.P.S., J.T.; Investigation: S.P.S.; Data curation: S.P.S., J.T.; Writing - original draft: S.P.S., J.T.; Writing - review & editing: S.P.S., R.S.J., S.E., E.E., J.T.; Visualization: S.P.S.; Project administration: S.P.S., J.T.

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