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1 **Article**

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3 **Effect of stimulation frequency on force, net power output and fatigue in mouse soleus muscle**

4 **in vitro**

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1   **Abstract:** The effects of electrical stimulation frequency on force, work loop power output and

2   fatigue of mouse soleus muscle were investigated in vitro at 35 ºC. Increasing stimulation frequency

3   did not significantly affect maximum isometric tetanic stress (overall mean ± SD, 205 ± 16.6

4   kN m^{-2} between 70 and 160 Hz) but did significantly increase the rate of force generation. The

5   maximal net power output during work loops significantly increased with stimulation frequency,

6   18.2 ± 3.7 W kg^{-1}, 22.5 ± 3.3 W kg^{-1}, 26.8 ± 3.7 and 28.6 ± 3.4 W kg^{-1} at 70, 100, 130 and 160 Hz

7   respectively. The stimulation frequency used affected the pattern of fatigue during work loop

8   studies. At stimulation frequencies of 100 Hz and 130 Hz there were periods of mean net negative

9   work during the fatigue tests due to a slowing of relaxation rate. In contrast mean net work

10   remained positive throughout the fatigue test when stimulation frequencies of 70 Hz and 160 Hz

11   were used. The highest cumulative work during the fatigue test was performed at 70 and 160 Hz,

12   followed by 130 Hz, then 100 Hz. Therefore, stimulation frequency affects power output and the

13   pattern of fatigue in mouse soleus muscle.

14

15   *Key words:* fatigue, force, mouse, power output, skeletal muscle, soleus, stimulation frequency,

16   work loop.

17

1 **Introduction**

2   During locomotion many muscles undergo cyclical length changes with phasic activation to

3 produce force and in some situations net power output (Dickinson et al. 2000; Kargo and Rome

4 2002). To maximize net power output during cyclical length changes the muscle must not only be

5 able to shorten rapidly while generating high force, but must also maintain force during shortening,

6 relax rapidly before subsequent lengthening and then activate rapidly near its maximal length ready

7 for high force generation during shortening (James et al. 1996; Josephson 1999; Caiozzo 2002). The

8 work loop technique has been used in vitro to subject muscle to cyclical length changes and phasic

9 stimulation to more closely simulate the type of activities that occur in vivo (Josephson 1985;

10 Josephson 1999; Caiozzo 2002). Previous studies have demonstrated the benefits of using the work

11 loop technique to investigate the cumulative mechanical effects of fatigue (decreased generation

12 and maintenance of force, increased relaxation time and decreased maximal velocity of shortening)

13 that lead to decreases in the net power output produced by mammalian muscle during cyclical

14 length changes (Askew et al. 1997; James et al. 2004).

15   Most in vitro studies of muscle performance have used maximal activation (including a relatively

16 high stimulation frequency). However, during many movements, muscles are used in vivo in a

17 submaximal manner, thus not all fibres are used for the specific task performed, and the recruitment

18 order of fibres is altered according to the activity undertaken (Wakeling 2005). The mean firing rate

19 during activity of soleus muscles in freely walking adult laboratory rats ranges from 12 to 45 Hz,

20 much lower than the approximately 100 Hz rate of stimulation required for maximal tetanic force

21 (Hennig and Lømo 1985; Gorassini et al. 2000). Therefore, it is unlikely that in vitro usage of high

22 stimulation frequencies will necessarily aid attempts to simulate in vivo action of muscles.

23   Previous research has demonstrated that under isometric conditions increased stimulation

24 frequency leads to increased tetanic force, until a plateau is reached, under both in vitro and in vivo

25 conditions (Buller and Lewis 1965; Rack and Westbury 1969; de Haan 1998; Mela et al. 2001).

26 Increased stimulation frequency under isometric conditions also increases the rate of force

1 generation, such that the maximal rate of force generation occurs at a higher stimulation frequency

2 than that eliciting maximal force (Buller and Lewis 1965; Rack and Westbury 1969; Bevan et al.

3 1992; de Haan 1998). Increased stimulation frequency also causes increased force and power to be

4 produced during shortening in isovelocity studies (de Haan 1998). Few studies have investigated the

5 effects of stimulation frequency on fatigue. The rate of fatigue elicited during isometric studies does

6 seem to be dependent on the stimulation frequency used (Bigland-Ritchie et al. 1979; Garland et al.

7 1988; Russ and Lovering 2006) but findings differ on the nature of this relationship.

8   The aim of the current study was to determine the effects of different stimulation frequencies on

9 force generation, work output and pattern of fatigue in mouse soleus muscles subjected to repeated

10 work loop cycles in vitro.

11

1 **Materials and Methods**

2 **Dissection**

3   Female white mice (strain CD1, Charles Rivers, Tranent, UK) aged between 8 and 10 weeks old

4 (body mass 29.5 ± 1.1g, $n = 34$ including the two muscles removed from analyses; see end of work

5 loop studies section) were used in the experimental procedure. Animals were cared for in

6 accordance with the Guide to the Care and Use of Experimental Animals (1993, available from the

7 Canadian Council on Animal Care) and the use of animals was reviewed and approved by the

8 Coventry University Ethics Committee. The animals were killed by cervical dislocation in

9 accordance with the British Home Office Animals (Scientific Procedures) Act 1986, schedule 1.

10   Soleus muscle was isolated from the right hind-limb and pinned out at approximately its resting

11 length at room temperature (19 to 21 °C), in oxygenated (95% O_2; 5% CO_2) Krebs-Henseleit

12 solution (composition, values in mM: NaCl 118; KCl 4.75; $MgSO_4$ 1.18; $NaHCO_3$ 24.8; KH_2PO_4

13 1.18; glucose 10; $CaCl_2$ 2.54; pH 7.55 at room temperature prior to oxygenation. For each soleus

14 muscle preparation, a small piece of bone was left attached at the end of both the proximal and

15 distal tendons. Aluminium foil clips were wrapped around the tendons at either end of the muscle.

16

17 **Isometric studies**

18   The soleus muscle preparation was attached via the aluminium foil clips to a force transducer

19 (UF1, Pioden Control Ltd, Barry Way, UK) at one end and a displacement transducer (V201, Ling

20 Dynamic System, Newport, UK) at the other end. For position detection a Linear Variable

21 Displacement Transducer (DFG 5.0, Solartron Metrology, Leicester, UK) was used. The

22 preparation was placed in a flow-through chamber containing circulating oxygenated Krebs-

23 Henseleit solution that was maintained at 35 °C via a water bath.

24   The muscle preparation was stimulated via parallel wire platinum electrodes (these were not in

25 contact with the muscle or nerve branch), while length was held constant, with 1.5 ms pulse width

26 stimuli to produce a series of isometric twitches. The stimulus amplitude (17 to 21 V) and muscle

length were optimized to yield the maximum isometric twitch force. The muscle length that corresponded to maximal isometric twitch force was measured via a microscope fitted with an eyepiece graticule and was defined as L_0 ($L_0 = 8.30 \pm 0.83$ mm; $n = 32$). Mean muscle fibre length was calculated as 85% of L_0 (James et al. 1995). Isometric tetanic force responses were elicited by subjecting the muscle to a 320 ms burst of electrical stimulation. In 8 muscles a number of stimulation frequencies (70, 100, 130 and 160 Hz) were used to determine the maximal isometric tetanic force. A recovery period of 5 minutes was allowed between each tetanus, to ensure full force recovery of the soleus muscle preparation (James et al. 1995). In all other muscles the stimulation frequency of 130 Hz was used to generate isometric tetanic force.

Work loop studies

The soleus muscle preparation was then subjected to sinusoidal length changes, using the work loop technique (Josephson 1985) to determine net power output. The stimulation amplitude that generated maximal twitch was used. Each soleus muscle preparation was subjected to four sinusoidal length change cycles using a total strain of 0.10 symmetrically around L_0 (lengthened from resting length to 5% longer than resting length, then shortened to 5% shorter than resting length, following a length change pattern of a sine wave) at a cycle frequency of 5 Hz. A strain of 0.10 has previously been estimated to be within both the range of strains that produce maximal power output in vitro at 5 Hz cycle frequency and within the range of strains used by soleus muscle during locomotion (James et al. 1995).

All muscles were electrically stimulated using a burst duration of 65 ms and a phase of -10 ms (stimulation started 10 ms prior to the muscle reaching maximal length and continued until 45 ms prior to shortest length) that was shown previously by James et al. 1995 to yield maximal power output at this cycle frequency. Muscle stimulation and length changes were controlled and monitored using custom written software (Testpoint, CEC, Norton, USA) via a D/A board (KPCI3108, Keithley Instruments, Cleveland, USA). A plot of force against length produced a

"work loop" that represented the net work done by the muscle during the length change cycle (Josephson 1985). The net work produced in the second work loop cycle was not significantly different ($p > 0.05$) from that found in cycle 3 or 4, therefore loop 2 was used as indicative of the ability of the muscle to produce work. In 8 muscles the effect of stimulation frequency on work loop power output was investigated by comparing stimulation frequencies of 70 Hz, 100 Hz, 130 Hz and 160 Hz, without changing any other strain or stimulation parameters. A set of control work loops were performed (using 130Hz stimulation frequency, 65 ms burst duration, stimulation phase of -10 ms, strain of 0.10, cycle frequency of 5 Hz) every fourth set of work loops to monitor any changes in the ability of the muscle to generate power over the time course of the experiment. The ability of each muscle preparation to generate power declined slightly over the time course of each experiment remaining higher than 90% of the maximal ability. The decrease in power output was due to a decrease in force production, therefore, all work loop power outputs were corrected assuming a linear decline between each control set of work loops (James et al. 1995).

Fatigue tests were performed at each of the stimulation frequencies under investigation (70 Hz, 100 Hz, 130 Hz and 160 Hz). Only one fatigue test was carried out per preparation (8 muscles were used for each stimulation frequency of fatigue test), at the end of each experiment, to ensure that metabolite levels were the same at the start of each fatigue test. A fatigue test consisted of subjecting the muscle to repeated cycles of work loops (with each loop subjected to a 5 Hz cycle frequency, strain of 0.10, 65 ms stimulus burst duration and stimulation phase of -10 ms) until 300 loops had been generated. Following this treatment the muscle preparation was allowed to recover for 30 minutes before the power output was measured over a series of four control loops. In any case where muscle power output had not recovered to a satisfactory level (to >85% of the initial power output within this 30 minute recovery period) the preparation was discarded. On this basis, all results from two of the soleus muscle preparations (from the initial 34) were discarded. Changes in muscle power output were monitored, during each fatigue test, as a function of time and as a function of cumulative work.

Muscle mass measurement and dimension calculations

At the end of each experiment, the tendons were removed from the muscle preparation, and excess Krebs-Henseleit solution was removed by blotting the muscle with tissue paper. Wet muscle mass was determined using an electronic balance (mass = 9.20 ± 1.14 mg; $n = 32$). Muscle power output and cumulative work were normalized to muscle mass. Muscle stress was calculated as force divided by mean muscle cross-sectional area (CSA). CSA was calculated from fibre length, muscle mass and an assumed muscle density of 1060 kg m^{-3} (Mendez and Keys 1960).

Statistical analysis

Values for mouse body mass, muscle mass, muscle fibre length, time to half peak tetanus, maximum isometric stress, maximum net power output (prior to fatigue test) and time required for net work loop power output to decline to 50% of initial power output during the fatigue test were analysed via single factor analysis of variance using SPSS statistical software (Version 13, SPSS Inc., Chicago, USA) using stimulation frequency as the independent variable. Tukey post hoc tests were used when $p < 0.05$ was obtained for the ANOVA. Data are expressed as mean $\pm$ SD.

1   **Results**

2   **Morphological measurements**

3   There were no significant differences between work loop fatigue treatment groups in measures

4   of body mass, muscle mass or muscle fibre length (ANOVA $p > 0.24$ and n = 8 in each case).

5   Therefore, morphology was not considered in further statistical analyses.

6

7   **Isometric properties**

8   Increased stimulation frequency caused time to half-peak tetanus (THPT) to significantly

9   decrease (Fig. 1; ANOVA $p < 0.001$), approximately 23%, between 70 and 130 Hz stimulation

10   frequencies (Tukey $p < 0.001$; Note that Tukey $p = 0.04$ between 70 and 100 Hz, $p = 0.18$ between

11   100 and 130 Hz), with little change from 130 to 160 Hz (Tukey $p = 0.84$). The mean maximum

12   isometric twitch stress for soleus muscle was 31.7 ± 3.2 kN m^{-2} ($n = 32$). The mean maximum

13   isometric tetanic stress for soleus muscle was 205 ± 16 kN m^{-2} ($n = 32$ at 130 Hz stimulation

14   frequency). There was no significant effect of stimulation frequency on maximal isometric stress

15   (Fig. 2; ANOVA $p = 0.73$).

16

17   **Effect of stimulation frequency on power output during work loop studies prior to fatigue**

18   In work loop experiments stimulation frequency had a significant effect on net work loop power

19   output prior to fatigue (ANOVA $p < 0.001$; Tukey $p < 0.001$ between 70 and 130 Hz and between

20   70 and 160 Hz, $p = 0.01$ between 100 and 160 Hz), increasing power output with increasing

21   stimulation frequency (Fig. 3). Power output increased by 57% when stimulation frequency was

22   increased from 70 to 160 Hz.

23

24   **Effect of stimulation frequency on fatigue during work loop studies**

25   Peak power output was achieved within the first 5 work loop cycles of the fatigue test in all

26   preparations at all stimulation frequencies (70 Hz, 100 Hz, 130 Hz and 160 Hz) under investigation

1   (Fig. 4). The time required for net work loop power output to decline to 50% of initial power output

2   was significantly dependent on the stimulation frequency used, with times of 14.4 ± 3.8 s at 70 Hz,

3   10.6 ± 2.3 s at 100 Hz, 8.9 ± 1.2 s at 130 Hz and 9.8 ± 1.9 s at 160 Hz (Fig. 4; ANOVA $p = 0.001$;

4   Tukey $p < 0.03$ between 70 Hz and all other stimulation frequencies).

5   At 70 Hz and 160 Hz stimulation frequency, the mean cumulative work continued to increase

6   throughout the fatigue test (Fig. 5). However, at stimulation frequencies of 100 Hz and 130 Hz

7   cumulative work increased to a maximum value, then decreased as net power output became

8   negative (Figs. 4, 5).

9

10   **Effect of fatigue on work loop shape**

11   The typical changes in work loop shape induced by fatigue at different stimulation frequencies

12   (70, 100, 130 and 160 Hz) during the fatigue test (work loops after 0.4 s, 5 s and 20 s) are shown in

13   figure 6. As the muscle fatigued, the net peak force that could be generated in the work loop was of

14   lower magnitude than in the previous work loop shown (e.g. higher force in the work loop after 0.2

15   s than after 5 s of the fatigue test). With further progression of fatigue (e.g. the work loop after 5 s

16   compared with after 20 s of the fatigue test), the force produced during shortening tended to decline

17   at a higher rate. In some instances (e.g. the work loop after 20 s during 100 and 130 Hz stimulation

18   frequency fatigue tests), the soleus muscle failed to relax completely during shortening, resulting in

19   an increase in force produced during lengthening and a subsequent increase in the work required to

20   re-lengthen the muscle. The work loop produced after 20 s in 100 and 130 Hz stimulation

21   frequencies typically resulted in a negative component in the work loop and in some cases in net

22   negative work (Figs. 4, 5, 6).

23

Discussion

Maximal mechanical properties of in vitro soleus muscle

Soleus muscle produced a maximal isometric tetanic stress of 205 ± 17 kN m^{-2} that is similar to the values of 212 kN m^{-2} and 224 kN m^{-2} previously found for mice at the same experimental temperature of 35 ˚C (James et al. 1995; Askew et al. 1997). The maximal power output determined using the work loop technique in this study was 28.6 ± 3.4 W kg^{-1} at a cycle frequency of 5 Hz, which is approximately 15% lower than in previous studies (James et al. 1995, 34 W kg^{-1}; Askew et al. 1997, 33.2 W kg^{-1}). This difference could be due to differences in strain or age of mice used resulting in muscle fibre type differences, and/or could be due to the shorter soleus muscle length and the lower stress in the present study (10% lower than the stress in Askew et al. 1997).

Effect of stimulation frequency on isometric stress

The maximum isometric stress produced by soleus muscle was independent of the stimulation frequencies used in this experiment (Fig. 2) indicating, that in mouse soleus muscle at 35 °C, the physiological maximal active state can be reached, at any stimulation frequency between 70 and 160 Hz. However, as in previous studies, as stimulation frequency was increased the rate of force generation also increased (Buller and Lewis 1965; Rack and Westbury 1969; Bevan et al. 1992; de Haan 1998).

Effect of stimulation frequency on work loop power output

The soleus muscle maximum power output, prior to fatigue, significantly increased with increasing stimulation frequency (Fig. 3). Cycle frequency (5 Hz) and maximal strain (0.10) were the same throughout the experiment, so any changes in power output (power output = [mean force × length change] ÷ time taken) were related to changes in force. A number of previous studies have modelled the changes in force output observed during work loop experiments (James et al. 1996; Askew and Marsh 1998; Curtin et al. 1998; Woledge 1998; Caiozzo and Baldwin 1997; Lichtwark

and Wilson 2005). The key findings of these studies are that the force produced at any instant during a work loop can be predicted from a combination of the force-velocity relationship, length-force relationship, activation state of the muscle and series elastic element stiffness. Figure 1 demonstrates that the rate of muscle force generation during a tetanus increases with increasing stimulation frequency, probably as a result of increased rate of calcium release. Therefore, our experimental manipulation of stimulation frequency is likely to have affected power output by altering the rate of activation of the muscle. The aforementioned modelling papers indicate that any increase in the rate of force generation during an isometric tetanus should translate into an increase in the rate of force increase during work loop studies.

Effect of stimulation frequency on muscle fatigue

All the changes induced by fatigue at the stimulation frequencies used were reversible, a key factor in ensuring that changes were due to peripheral fatigue and not due to muscle damage (James et al. 2004; Askew et al. 1997). The soleus muscle recovered rapidly to attain almost pre-fatigue performance within 30 minutes of the end of the fatigue test.

The total cumulative work achieved was initially greater at higher stimulation frequency due to higher stimulation frequencies resulting in higher net power output per loop (Figs. 4, 5). However, as the fatigue test progressed the work produced decreased more rapidly at 100 Hz and 130 Hz stimulation frequencies, with corresponding changes in work loop shape. During the fatigue test peak work loop force decreased at all stimulation frequencies (Fig. 6). However, at both 100 Hz and 130 Hz stimulation frequencies the work loop obtained after 20 s of the fatigue test had a large "negative work" component which can be attributed to the prolongation of relaxation that increases the work required to extend the muscle to its initial length (lengthening work). Increases in lengthening work could be associated with a range of intracellular factors that affect the rate of relaxation, via their effect on $[Ca^{2+}]_i$ (Westerblad and Allen 1994). Prolongation of relaxation has been observed in other studies where a large number of cycles of work loops have been performed

1 (Stevens and Syme 1993; Askew et al. 1997; James et al. 2004). In the present study 160 Hz

2 stimulation frequency resulted in higher peak work loop force and higher force to be maintained

3 during shortening when compared with other stimulation frequencies (Fig. 6). The reasons

4 underlying improved fatigue resistance at 160 Hz stimulation frequency remain unclear.

5

6 **Relevance of the present study to in vivo conditions**

7 During locomotion in the mouse, the soleus muscle is believed to be active during both

8 shortening and near isometric phases of the movement (James et al. 1995). Therefore, the

9 experimental conditions used in this study are a simplification of in vivo conditions (James et al.

10 1995). Variation in muscle length change waveform complexity can have large effects on muscle

11 force and power-output (Askew and Marsh 1998). Therefore, further studies need to be performed

12 to determine in vivo strain waveforms for mouse muscle and to subject in vitro muscle to these

13 strain waveforms to simulate in vivo function.

14 During locomotion the activation patterns imposed on mouse soleus will likely be far more

15 complex than those used in the present study, but further studies need to be performed using

16 electromyography to determine the in vivo muscle activation patterns for running mice. However,

17 we can use the in vitro data in the present study to make some predictions about how muscle

18 activation might alter either to maximise performance during sprinting or to enhance endurance

19 during prolonged in vivo activities. During activities such as sprinting, where maximal acceleration

20 is important, it is likely that higher motor unit firing rates would be used in vivo to maximise the

21 rate of force generation. In contrast, during endurance type activities lower motor unit firing rates

22 are likely to be used to increase fatigue resistance. Previous work on walking rats has demonstrated

23 that mean firing rates in soleus muscle are far lower than those required to produce maximal tetanic

24 force (Hennig and Lømo 1985; Gorassini et al. 2000). However, little is known about firing rates in

25 running rodents, so it is difficult to determine how relevant the higher stimulation frequencies used

26 in the present study are in relation to locomotory firing rates. In the present study stimulation of

soleus muscle at submaximal levels (e.g. 70 Hz) delayed the onset of fatigue. Lower stimulation frequencies than those generating maximal force are likely to be regularly used in vivo to limit the decrease in power output due to slowing of relaxation rate during the fatigue test in the present study. Under in vivo conditions the burst duration, frequency and intensity of 'stimulation' could be modulated (Wakeling 2005), possibly with changes made in response to the alteration in force output of the muscle during fatigue. In vitro studies have also demonstrated that isometric peak force generation is higher and fatigue resistance is greater when the inter-pulse interval of stimulation is relatively short at the start of stimulation compared to when a constant stimulation frequency is used (Bevan et al. 1992; van Lunteren and Moyer 2004). In vitro experiments on locust flight muscle have demonstrated that a reduction in the stimulus burst duration during work loops decreases the initial power output, but increases efficiency and allows power output to be maintained for a longer period of time (Josephson and Stevenson 1991). Such a reduction in stimulus burst duration would probably have reduced the negative components of the work loop cycles observed later in the fatigue test in the present study. Rotation of stimulation pulses round the motor units in a muscle (asynchronous stimulation) can also result in a higher force than when synchronous stimulation of the whole muscle at the same stimulation frequency is used, especially when length is lower than L_0 and lower stimulation frequencies are used (Rack and Westbury 1969; Sandercock 2006). Supramaximal firing of soleus muscle in vivo, is unlikely to be a common phenomenon, since for maximal economy, the muscle should be stimulated at a frequency not greater than that required for full activation.

In summary, there was no effect of electrical stimulation frequency on the maximum isometric tetanic stress produced by soleus muscle in the present study. The net power output, generated during work loop studies, significantly increased as stimulation frequency was increased from 70 Hz to 160 Hz. The stimulation frequency used affected the pattern of fatigue during work loop studies. At stimulation frequencies of 100 Hz and 130 Hz there were periods of mean net negative work during the fatigue test, probably due to a slowing of relaxation rate. In contrast mean net work

1   remained positive throughout the fatigue test when stimulation frequencies of 70 Hz and 160 Hz

2   were used. Therefore, electrical stimulation frequency has a direct effect on both power output and

3   the overall pattern of fatigue in mouse soleus muscle.

4

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17

Figure captions

Fig. 1. Effect of stimulation frequency (70, 100, 130 and 160 Hz) on time to half peak tetanus

(THPT) of mouse soleus muscle prior to fatigue at 35 ℃. Data represent mean ± SD ($n = 8$).

Fig. 2. Effect of stimulation frequency (70, 100, 130 and 160 Hz) on maximum isometric tetanic

stress of mouse soleus muscle prior to fatigue at 35 ℃. Data represent mean ± SD of muscle force

values normalised to muscle cross-sectional area ($n = 8$)

Fig. 3. Effect of stimulation frequency (70, 100, 130 and 160 Hz) on net work loop power output

for mouse soleus muscle prior to fatigue at 35 ℃. Data represent mean ± SD of power values

normalised to muscle mass ($n = 8$)

Fig. 4. Effect of stimulation frequency on fatigue of mouse soleus muscle power output during

work loop studies at 35 ℃. Data represent mean ± SD of power values normalised to muscle mass

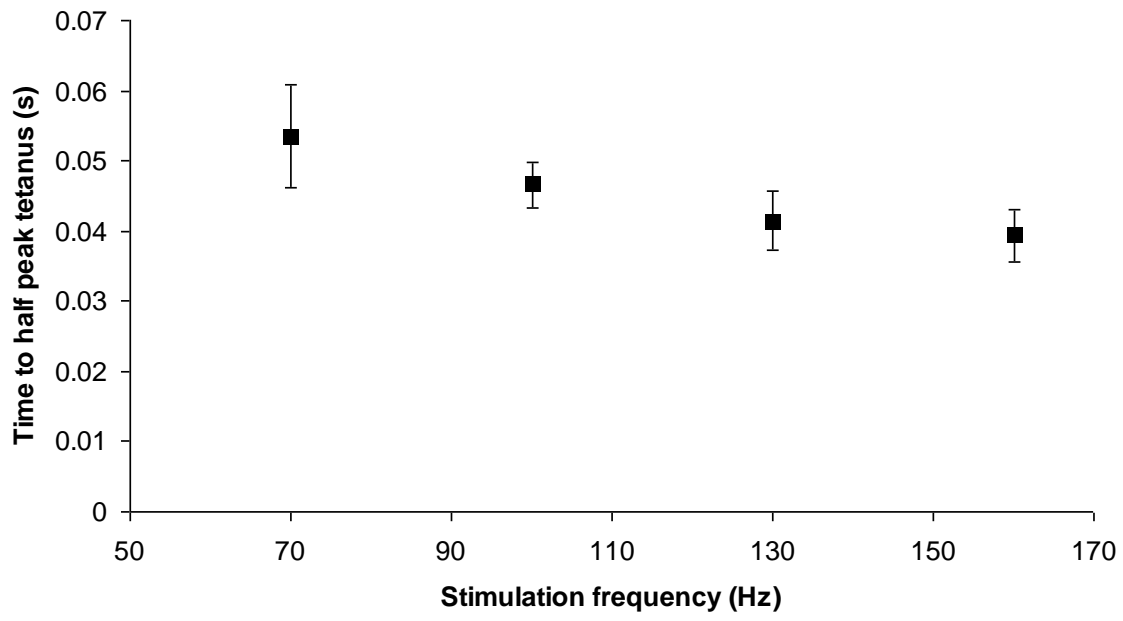($n = 8$) over 20 s of the fatigue tests. For clarity not all standard deviation bars are shown.

Fig. 5. Effect of stimulation frequency on the relationship between mouse soleus net muscle power

output and cumulative work produced during work loop studies at 35 ℃. Values represent mean ±

SD of power values normalised to muscle mass ($n = 8$) over 20 s of the fatigue tests. For clarity not

all standard deviation bars are shown.

Fig. 6. Typical changes in work loop shape at different stimulation frequencies (70 Hz, 100 Hz, 130

Hz and 160 Hz) over 20 s of the fatigue tests of mouse soleus muscle at 35 ℃. For each work loop

stress is plotted against strain.

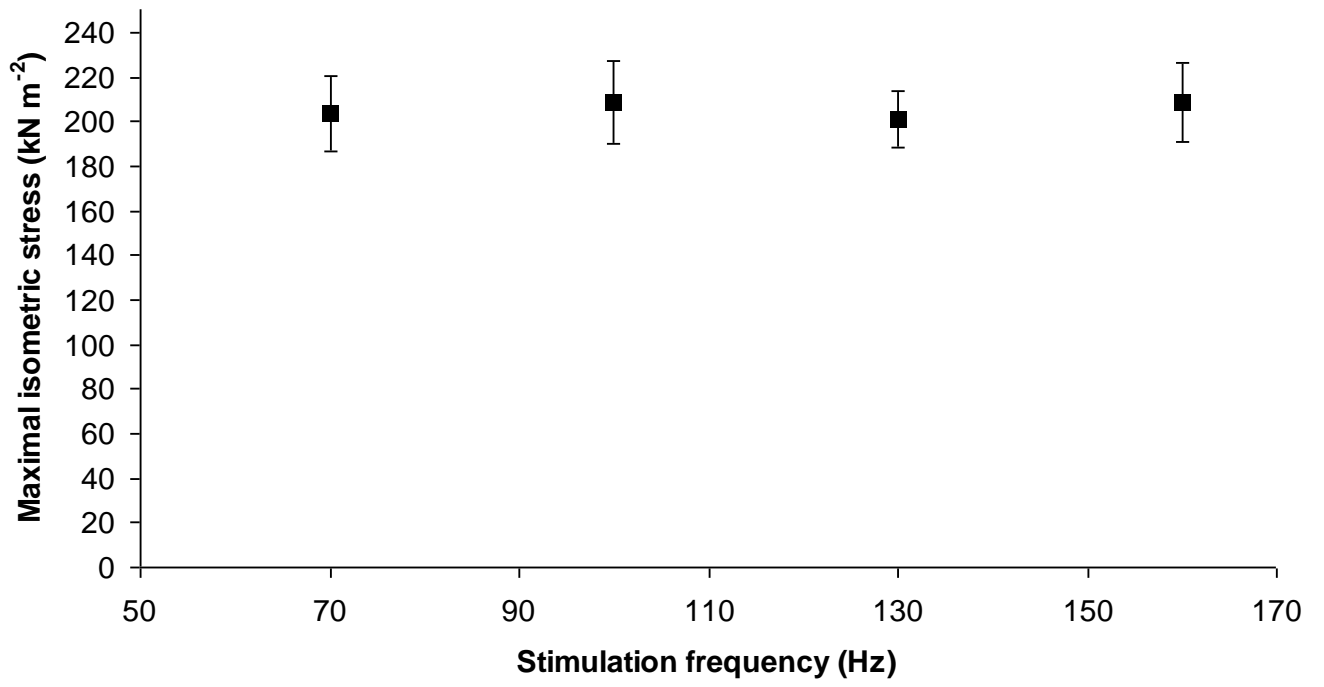1 **Figure 1.**

2

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1   **Figure 2.**
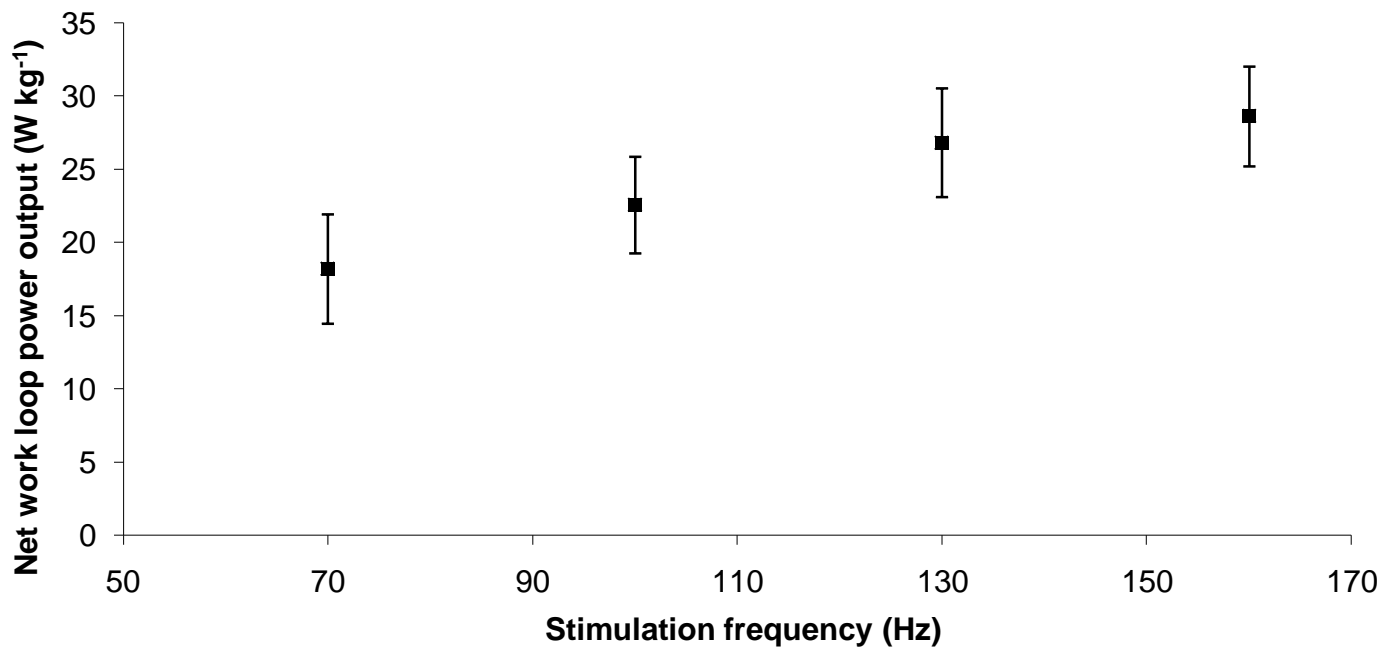
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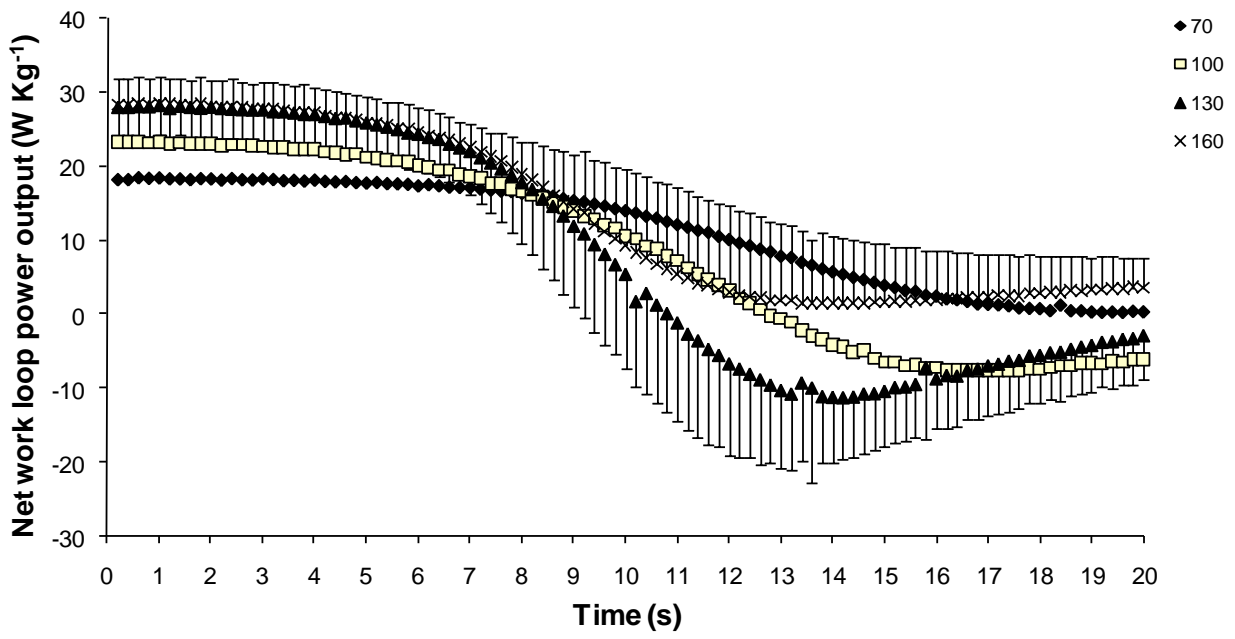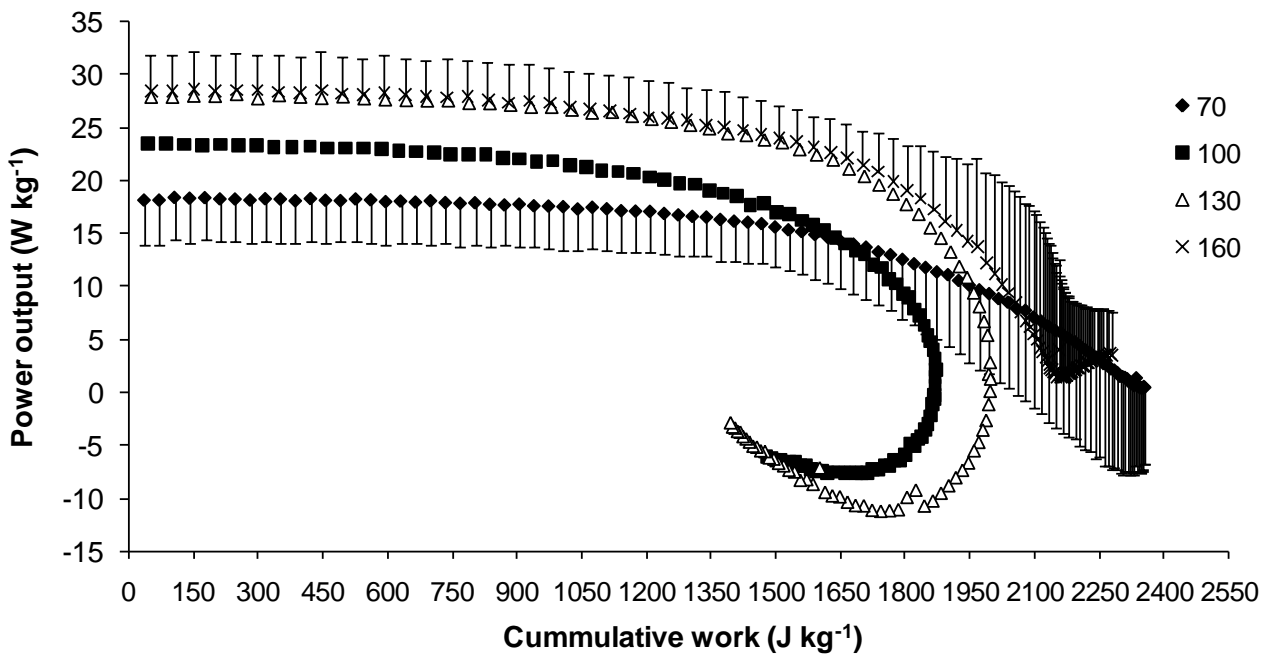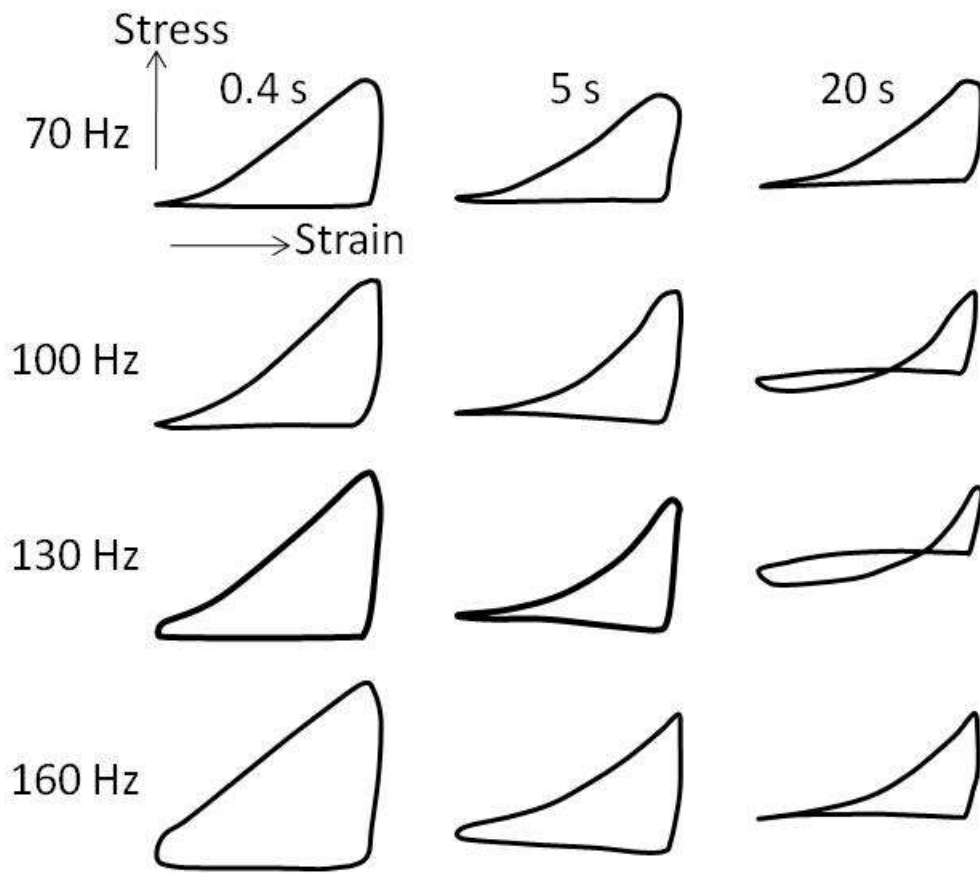1   **Figure 3.**

1 **Figure 4.**

2

Figure 5.

1   **Figure 6.**

2