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SHORT COMMUNICATION

Factors associated with electrical stimulation-induced performance fatigability are dependent upon stimulation location

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Abstract

Neuromuscular electrical stimulation (NMES) is increasingly viewed as a central tenet to minimise muscle loss during periods of disuse/illness – typically applied directly over a muscle belly. Peripheral nerve stimulation (PNS) is afforded less attention, despite providing a more global contractile stimulus to muscles. We investigated NMES versus PNS in relation to performance fatigability and peripheral contributions to voluntary force capacity. Two fatigue protocols were assessed separately: (1) over-quadriceps NMES and (2) peripheral (femoral) nerve stimulation (PNS). Before and after each session, a maximal voluntary contraction (MVC) was performed to assess force loss. Knee-extensor force was measured throughout to assess contractile function in response to submaximal electrical stimulation, and M-wave features quantified myoelectrical activity. NMES and PNS induced similar voluntary (MVC, NMES: $-12 \pm 9\%$, PNS: $-10 \pm 8\%$, both $P < 0.001$) and stimulated (NMES: $-45 \pm 12\%$, PNS $-27 \pm 27\%$, both $P < 0.001$) force reductions. Although distinct between protocols, myoelectrical indicators of muscle recruitment (M-wave area and amplitude) and nerve conduction time did not change throughout either protocol. Myoelectrical propagation speed, represented as M-wave duration, and the delay before muscle relaxation began both progressively increased during NMES only ($P < 0.05$ and $P < 0.001$, respectively). NMES myoelectrical changes suggested performance fatigability, indicating activation of superficial fibres only, which was not observed with PNS. This suggests PNS recruits a wider pool of muscle fibres and motor units and is a favourable alternative for rehabilitation. Future work should focus on implementing PNS interventions in clinically relevant scenarios such as immobilisation, care homes and critical illness.

KEYWORDS
electromyography, fatigue, myoelectrical characteristics, neuromuscular electrical stimulation, skeletal muscle

1 | INTRODUCTION

Electrical muscle stimulation is a commonly applied rehabilitation strategy aimed at minimising loss of strength and muscle mass during periods of disuse (Guo et al., 2020; Liu et al., 2020), especially important in the older population, due to the accelerated loss of muscle mass, i.e., sarcopenia (Wilkinson et al., 2018). Although neuromuscular electrical stimulation (NMES) produces notable benefits such as recovering muscle mass and function following reduced use (Enoka et al., 2020), protocols are highly heterogeneous rendering measurable outcomes difficult to compare (Trethewey et al., 2019).

Direct muscle stimulation results in a non-physiological recruitment order of motor units (MUs) (Henneman, 1957), reported as a reversal of normal recruitment (Kubiak et al., 1987), in which the faster-contracting type IIa/x-associated MUs are activated first (Trimble & Enoka, 1991), although more likely in a non-selective, spatially fixed and temporally synchronous order (Bickel et al., 2011), dependent on proximity to the stimulating electrode (Mesin et al., 2010). Conversely, peroneal nerve stimulation has been shown to recruit equally between superficial and deep MUs, suggesting nerve stimulation may follow a different recruitment pattern to voluntary contractions and NMES applied over the muscle (Okuma et al., 2013). Furthermore, quadriceps NMES has been shown to produce a linear recruitment curve, suggesting a random recruitment pattern from deeper MUs as intensity increases, while femoral peripheral nerve stimulation (PNS) produced a sigmoid curve, suggesting a tightly packed axonal distribution with a greater spatial uniformity to MU activation (Rodriguez-Falces et al., 2013). The different order of recruitment from nerve and muscle stimulation would be expected to produce a different extent of fatigue development over prolonged protocols, with larger MUs more likely to be composed of type IIa/x fibres and thus more fatigable. However, to date, muscle and nerve stimulation have not been compared using fatiguing protocols.

Fatigue can be defined as a psychophysiological disabling symptom wherein both physical and cognitive functions are limited by performance and perceived fatigability characteristics (Enoka & Duchateau, 2016). Although fatigue may not be critical for muscle adaptation, it remains a useful stimulus for it (Folland et al., 2002). Fatigue can be further classified as perceived fatigability or performance fatigability (Enoka & Duchateau, 2016), the former relating to body homeostasis and psychological state while the latter refers to changes in contractile function and muscle activation. Acute decrements of contractile function are largely dependent on calcium kinetics (Cheng et al., 2018) as well as force capacity, blood flow and cellular metabolism. Electrical simulation protocols target performance fatigability and rely less on perceived fatigability, allowing the elicited activity to extend further than perceived fatigue. This is of particular use in situations where activity is reduced due to high perceived fatigability or to the extreme where ambulation is not possible such as intensive care units (Dirks et al., 2015).

New Findings

- **What is the central question of this study?**
  How does peripheral nerve stimulation (PNS) compare with neuromuscular electrical stimulation (NMES) used clinically to reduce muscle atrophy?
- **What is the main finding and its importance?**
  NMES resulted in progressive increases in M-wave duration and delay of muscle relaxation throughout a single stimulation protocol, findings not observed with PNS. This suggests PNS recruits from a wider pool of muscle fibres/motor units, providing a more favourable alternative to NMES for rehabilitation intervention.

Historically, the M-wave has been used in the measurement of peripheral fatigue, with its properties being shown to change during the activation of MUs using transcutaneous electrical stimulation at varying stimulation intensities (Farina et al., 2004). The M-wave represents a summation of the detected myoelectrical activity within recording range from a stimulated contraction (i.e., all muscle fibres that were depolarised following stimulation; Rodriguez-Falces & Place, 2017*a*), and therefore does not represent total muscle size/depth (Piasecki et al., 2018). Current evidence suggests the positive peak, or second phase, of the M-wave is highly susceptible to alteration based on external factors such as muscle fascicle pennation angle and tendon length (Rodriguez-Falces & Place, 2017*b*). Therefore, the negative peak (first phase) should be measured individually to provide an accurate representation of muscle myoelectrical activity. Changes in M-wave characteristics indicate changes in sarcolemmal excitability, which represents changes in stimulated contractile force. Therefore, it should be noted the M-wave cannot account for additional fatigue-related parameters such as reduced Ca^{2+} reuptake and sensitivity (Enoka & Duchateau, 2016).

A collection of studies have compared short, acute stimulation protocols to investigate peripheral and central contributions to torque generation (Baldwin et al., 2006; Bergquist et al., 2011, 2012) and have reported inconsistent results across muscles and modalities regarding peripheral and central input to torque. To our knowledge, no studies have compared NMES and PNS protocols and the impact they have on performance fatigability by considering myoelectrical and mechanical aspects of voluntary and involuntary force decrement. Therefore, the purpose of the present study was to investigate the impact of identical fatiguing protocols elicited via stimulation of the femoral nerve or muscle belly, on vastus lateralis (VL) myoelectrical, and quadriceps mechanical markers of performance fatigability. We hypothesised that PNS would induce a greater level of performance fatigability than NMES, which would be reflected by greater

voluntary force decrements, greater reduction in contractile function shown by greater loss of stimulated force, and larger differences in M-wave characteristics indicating changes in sarcolemmal excitability.

2 | METHODS

2.1 | Ethical approval

This study was approved by the local University Research Ethics Committee (ethics code: 523-2002) and conformed with the *Declaration of Helsinki* except for registration in a database. Participants were recruited locally from the University of Nottingham via advertisement posters. After providing written informed consent to participate in the study, participants were screened for eligibility. All included participants fulfilled recruitment criteria of being healthy, recreationally active and of normal weight or overweight (i.e., non-obese). Once eligibility was confirmed, participants were invited to the research laboratory for two visits separated by an average of 7 days to ensure muscle function was not impaired from the previous session. Participants were requested to refrain from vigorous exercise 3 days prior to each visit.

2.2 | Muscle ultrasound

For characterisation purposes, an ultrasound scan of the VL ($n = 13$) was performed on each participants' first visit using an ultrasound probe (LA523 probe, frequency range 26–32 Hz, and MyLab™50 scanner, Esaote, Genoa, Italy) to determine muscle cross-sectional area (CSA) at the mid-belly. With participants lying supine, the mid-belly of the muscle was identified as the mid-point between the greater trochanter and the mid-point of the patella. Medial and proximal borders of the VL were identified from the points at which the aponeurosis intersected with that of the m. vastus intermedius before three axial plane images were collected. Images were subsequently analysed using ImageJ (Laboratory of Optical and Communication, University of Wisconsin-Madison, WI, USA) to allow CSA quantification.

2.3 | Maximal voluntary contraction

Participants were seated in a custom-built dynamometer with hip and knee angles secured at 100° and 90°, respectively, using a waist belt and an ankle strap to secure the lower leg. Following a standardised warm-up of five mid-intensity contractions, verbal encouragement was given while participants performed an isometric knee extensor maximal voluntary contraction (MVC). After a rest of 30 s, a second MVC was performed to ensure maximal force was being achieved. If the second attempt was >5% different from the first, a third attempt was made and the highest recorded.

2.4 | Surface electromyography and force recording

The E1 electrode was placed over the identified motor point with E2 electrode placed over the patellar tendon (disposable self-adhering Ag–AgCl electrodes; 95 mm^2; Ambu Neuroline, Baltorpbakken, Ballerup, Denmark) in a bipolar configuration (Piasecki et al., 2016; Swiecicka et al., 2019). A ground electrode (E0) was placed just above the reference electrode (Ambu Neuroline Ground). Sampling of surface electromyography (EMG) signals was performed at 10 kHz then bandpass filtered at 5 Hz to 5 kHz (1902 amplifier, Cambridge Electronics Design Ltd, Cambridge, UK). Force transducer signals were sampled at 100 Hz. EMG signals were digitized (CED Micro 1401; Cambridge Electronic Design) with Spike2 (version 9.09a, Cambridge Electronic Design) software used to display the signal in real-time on screen.

2.5 | Electrically stimulated fatigue protocol

All participants received two different stimulation modalities in the same format to induce performance fatigue. Electrical stimulation was delivered over the femoral nerve (PNS) during one visit and over the quadriceps (NMES) on the other visit. The order of delivery was randomised. To perform PNS, large stimulating electrodes (ValuTrode cloth electrodes, 8 × 13 cm; Axelgaard Manufacturing Co., Fallbrook, CA, USA) were placed in the right inguinal fold (cathode) and on the right gluteal muscles (anode) (Piasecki et al., 2016; Swiecicka et al., 2019). For NMES, the electrodes were placed over the right quadriceps, centred 1 cm apart, proximal (cathode) and distal (anode) of the midline of the femur measured from the greater trochanter to the midline of the patella. The stimulation protocol used was based on previously published literature (Mcphee et al., 2014; Wüst et al., 2008). In brief, a 30 Hz pulse was applied at 400 V, 25 μS pulse width with a current to elicit an involuntary contraction of 30% MVC. Stimulation was carried out using a Digitimer DS7AH stimulator (Digitimer Ltd, Welwyn Garden City, UK). Once the appropriate current had been determined, 30 pulses were delivered 1 s in length with 1 s intervals between each pulse (Figure 1). Following the 2-min test, an MVC was performed within 10 s to measure performance fatigability. Discomfort was measured following each test using a visual analogue scale (VAS) from 0 to 10 with 0 being no discomfort and 10 being maximal discomfort.

2.6 | Neuromuscular parameters

Voluntary and involuntary force were recorded via a force transducer with raw data extracted using Spike2 (version 9.09a). Relaxation delay (RD) was measured from the peak of the final M-wave in a train produced by each 30 Hz pulse to the last turning point in the force trace before it began to decline (Figure 1a). M-wave parameters of negative peak area, duration and amplitude, along with nerve conduction time,

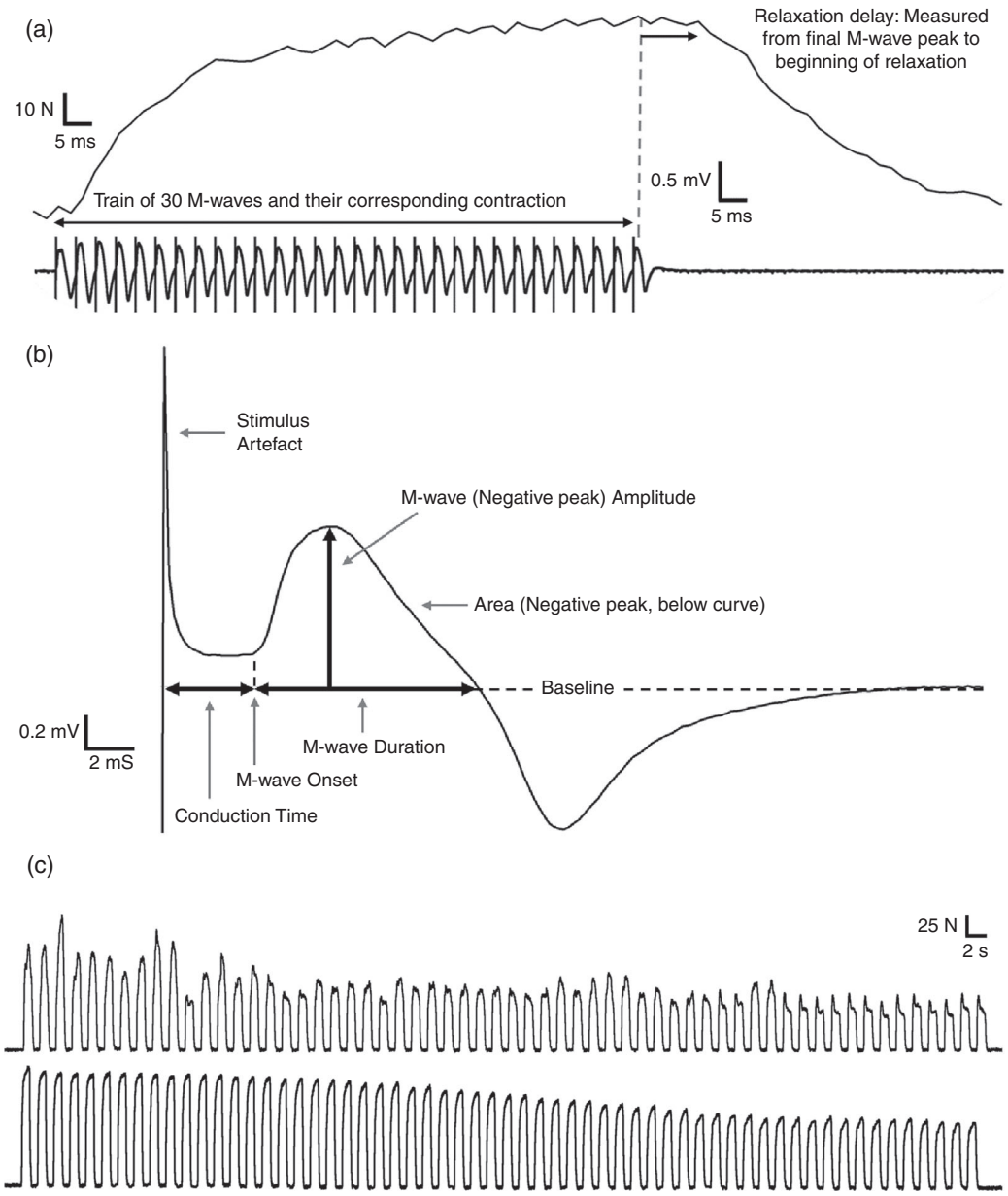

FIGURE 1 (a) A train of 30 consecutive M-waves and the corresponding contraction elicited by a single 30 Hz pulse of neuromuscular electrical stimulation, followed by relaxation delay measurement. This parameter was measured in time from the peak of the final M-wave in the train to the final turning point before a steep decline in force. Upper channel: force, measured in newtons, by time. Lower channel: electrical activity, measured in mV, by time. (b) Parameters of a single M-wave: M-wave negative peak area, amplitude and duration, and nerve conduction time; area measured from M-wave onset to intersection with baseline; amplitude measured from baseline to negative peak; duration measured as time from onset to intersection with baseline; nerve conduction time measured as time from stimulus artefact to M-wave onset. (c) Force traces from a single individual: variable force with peripheral nerve stimulation (upper) and progressive force decline with direct muscle stimulation (lower)

were measured from the final three M-waves in each train from the 1st, 15th, 30th, 45th and 60th contractions (Figure 1b).

2.7 | Statistical analysis

Data were analysed using GraphPad Prism version 8.4.1 (GraphPad Software, La Jolla, CA, USA). Student's paired *t*-test was used to assess stimulation current and VAS. All other variables were assessed using repeated measures two-way analysis of variance with Šidak's *post hoc* analysis. Two within-subject factors were assessed: time (pre and post) and condition (PNS and NMES). Data are expressed as means ± standard deviation. Statistical significance was accepted at $P < 0.05$. Due to large variability in individual baseline values, percentage change is presented in M-wave characteristics and RD for clarity of data display.

TABLE 1 Descriptive characteristics of participants

Characteristic	Value
Age (years)	27.06 (4.88)
Height (cm)	172.28 (11.05)
Weight (kg)	70.47 (17.83)
BMI (kg/m^2)	23.26 (3.84)
Vastus lateralis cross sectional area (cm^2)	23.18 (7.95)

Data are the mean and SD (n = 16, 8 male). BMI, body mass index.

3 | RESULTS

3.1 | Participant characteristics

Sixteen participants (eight male) completed the study. Participant characteristics are displayed in Table 1.

3.2 | Stimulation intensity and discomfort

Stimulation current (mA) required to elicit an involuntary contraction of 30% MVC was greater in NMES than PNS (132.4 ± 55 vs. 90.1 ± 25 mA, $P < 0.001$). Correspondingly, participants reported greater discomfort during NMES than PNS (5.3 ± 1.8 vs. 3.3 ± 1.6, $P < 0.001$).

3.3 | Performance fatigability

MVC decreased following both PNS (459.9 ± 184.7 vs. 411.2 ± 166.9 N, $P < 0.001$) and NMES (474.7 ± 188.1 vs. 412.5 ± 153.2 N, $P < 0.001$), with no significant interaction (−13.56, 95% CI −36.81 to 9.677, $P = 0.23$, Figure 2a). Similarly, stimulated force also decreased from the start to the end of the fatigue protocol in both PNS (136.0 ± 60.5 vs. 90.6 ± 38.9 N, $P < 0.001$) and NMES (133.7 ± 48.1 vs. 72.5 ± 26.9 N, $P < 0.001$) conditions, with no significant interaction (53.28, 95% CI 35.9–70.7, $P = 0.16$ Figure 2b).

3.4 | Relaxation delay

There was a significant interaction for both time and stimulation modality on RD ($P < 0.001$, Figure 2c). Šídák's *post hoc* analysis demonstrated that NMES increased RD throughout the stimulation, with RD significantly longer than with PNS at contraction 30 ($P < 0.01$), 45 and 60 ($P < 0.001$). This increase in RD was progressive throughout NMES, with RD at each contraction being greater than the first to an increasing degree (between contractions 1 and 15, $P < 0.01$; between contractions 1 and 30, 1 and 45 and 1 and 60, all $P < 0.001$).

3.5 | M-wave characteristics

M-wave characteristics are each reported as the average value of the last three recorded M-waves (from 30 in each pulse) at contractions 1, 15, 30, 45 and 60. Data are shown as a percentage of baseline with contraction 1 values set at 100%. For M-wave area, there was a significant interaction effect between time and condition (−12.3, 95% CI −24.5 to −0.11, $P < 0.05$). When analysed separately, M-wave area was greater for NMES than PNS at each contraction (all $P < 0.001$, Figure 3a).

M-wave amplitude analysis revealed a significant effect of condition with a moderate effect size (partial $\eta^2 = 0.13$, $P < 0.05$) and no significant interaction between conditions (−1.99, 95% CI −3.8 to −0.14, $P = 0.71$). Following *post hoc* analysis, M-wave amplitude was greater for NMES than PNS overall ($P < 0.05$, Figure 3b); this was apparent for each of the five contraction times (all $P < 0.001$, Figure 3b).

M-wave duration analysis showed a significant interaction effect (2.07, 95% CI 1.02–3.12, $P < 0.01$). When analysed separately, M-wave duration was lower with NMES than PNS at contractions 1, 15 (both $P < 0.001$), 30 ($P < 0.05$) and 60 ($P < 0.01$). A progressive increase in

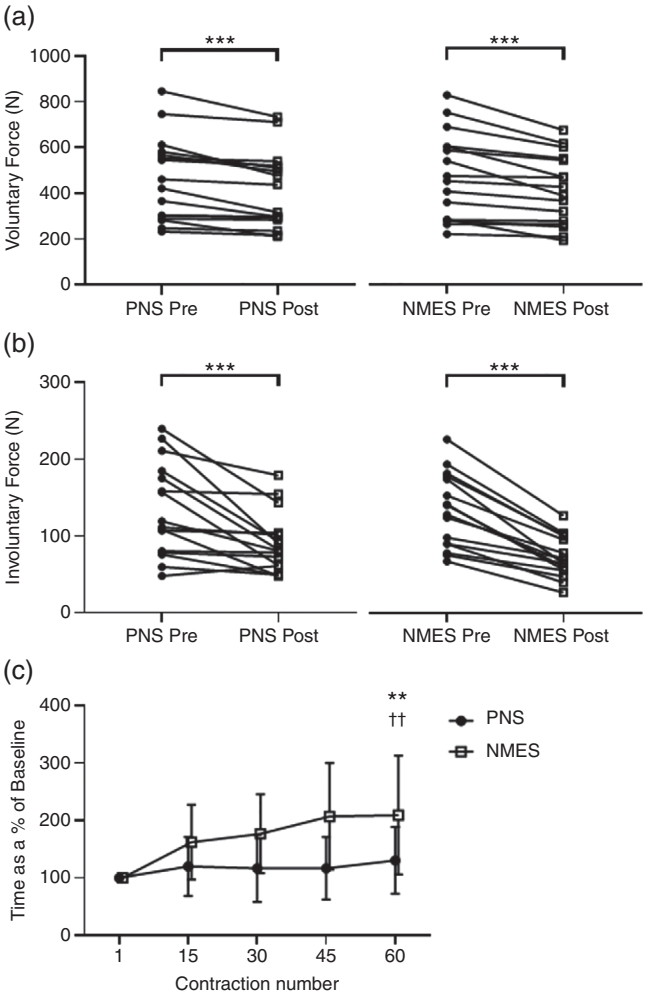

FIGURE 2 (a, b) Voluntary (a) and stimulated (b) force before and after peripheral nerve (PNS) and neuromuscular electrical (NMES) stimulation. Analysis via repeated measures two-way analysis of variance with Šídák's *post hoc* analysis. ***$P < 0.001$. (c) Relaxation delay with PNS (●) and NMES (□). Analysis via repeated measures two-way analysis of variance with Šídák's *post hoc* analysis. **$P < 0.01$ between stimulation modality; ††$P < 0.01$ vs. contraction 1 for NMES only

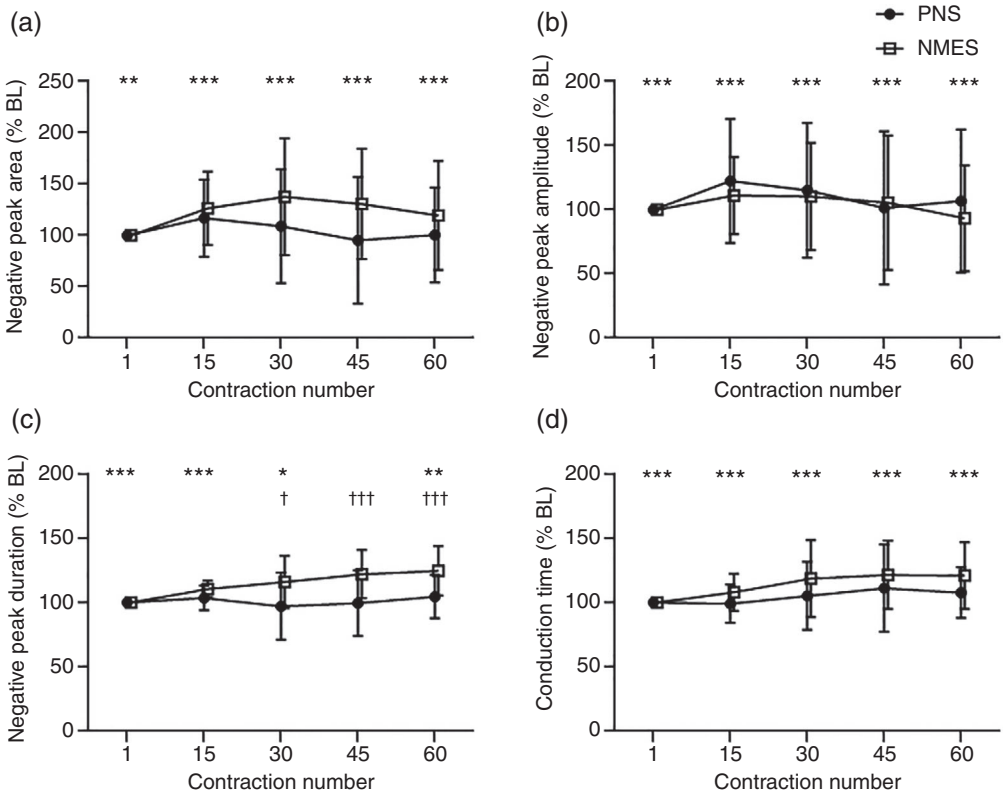

FIGURE 3 M-wave characteristics of peripheral nerve (PNS, ●) and neuromuscular electrical (NMES, □) stimulation. (a) Negative peak area, (b) negative peak amplitude, (c) negative peak duration, and (d) conduction time from stimulation to M-wave onset. Analysis via repeated measures two-way analysis of variance with Šidak's *post hoc* analysis. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ between stimulation modality; †$P < 0.05$, †††$P < 0.001$ vs. contraction 1 for NMES only

M-wave duration was seen with NMES only (from contraction 1 to 30 ($P < 0.05$), 1 to 45 ($P < 0.001$) and 1 to 60 ($P < 0.001$)) (Figure 3c).

As expected, based on stimulation sites, there was a large main effect of condition for nerve conduction time (partial $\eta^2 = 0.32$, $P < 0.001$), with nerve conduction time lower with NMES than PNS for all contractions ($P < 0.001$) (Figure 3d). No significant interaction was present (1.94, 95% CI 1.08–2.80, $P = 0.9$).

As this was a mixed-sex sample, secondary analyses using three-way ANOVAs (factors: time, condition, sex) were performed to investigate the influence of sex. No three-way interaction was revealed for MVC ($P = 0.17$) and involuntary force ($P = 0.38$) decline, or RD ($P = 0.18$). Similarly, there was no three-way interaction for M-wave amplitude ($P = 0.07$) and duration ($P = 0.68$) along with nerve conduction time ($P = 0.75$). However, there was a significant three-way interaction for M-wave area ($P < 0.05$).

4 | DISCUSSION

These data demonstrate that fatiguing electrical stimulation protocols applied via the motor nerve (PNS) or muscle (NMES) result in similar voluntary and involuntary force decrements. However, PNS was more tolerable and required a reduced stimulation intensity compared to NMES. Myoelectrical activity, as assessed by the M-wave, showed no progressive change across fatiguing contractions with PNS. Notably, M-wave duration and RD progressively increased throughout the protocol with NMES, with little change across other variables.

4.1 | Voluntary and stimulated force

The level of performance fatigability shown here is similar to that reported in previous studies that have used the same protocol, when applied over the muscle only (Mcphee et al., 2014; Wüst et al., 2008). Although we report no statistical difference in the involuntary force reductions elicited by PNS and NMES, the two methods induced different force profiles throughout the protocol. This is evidenced by similar group mean force values at each contraction (NMES, 103 N; PNS, 110 N) yet a two-fold larger SD in NMES (NMES, 21.2 N; PNS, 9.1 N). Put simply, the gradual stepdown in force with NMES was not observed with PNS, with the latter remaining close to baseline throughout (example in Figure 1c). This somewhat erratic pattern of force with PNS is indicative of variable MU recruitment, occurring in a non-selective and non-physiological manner (Bickel et al., 2011) Furthermore, this is consistent with the findings of Okuma et al. (2013) that peroneal nerve stimulation recruited equally from deep and superficial MUs.

4.2 | Relaxation delay

Slowing of relaxation was first shown to occur in fatigued single fibres of mouse muscles following a lack of available Ca^{2+} ions, thought to be caused by sarcoplasmic reticulum calcium pump (SERCA) impairment (Westerblad & Allen, 1993). Most, if not all studies investigating the relaxation of human skeletal muscle have focused on the time taken for the muscle to relax, measured from the beginning of force reduction to force returning to baseline, rather than on the time delay before reduction of force takes place, with the latter of these measures better representing an impairment of muscle fibre relaxation. Indeed, to our knowledge, this is the first study to show a delay of relaxation (temporal difference of M-wave and force decrease) in fatigued muscle following NMES applied to the muscle belly. This is suggestive of NMES recruiting from a select group of muscle fibres which are subsequently fatiguing, with no potentiation from muscle fibres which do not receive the stimulus. Furthermore, the lack of this observation in PNS further supports the suggestion that this stimulation modality recruits from a wider pool of muscle fibres than NMES.

4.3 | M-wave characteristics

The key finding from assessing M-wave parameters was the progressively increasing M-wave duration observed in NMES which was absent in PNS. This finding is in agreement with previous studies which have applied sustained stimulation to the muscle belly (Farina et al., 2004). The lack of change in M-wave duration with PNS again supports the theory that PNS is stimulating a wider pool of muscle fibres. The increased M-wave duration with NMES could be caused by a localised fatigue of a select number of superficial muscle fibres and a dysregulation of excitation–contraction coupling, in particular reduced Na^+,K^+-ATPase activity (McKenna et al., 2008). This reduction combined with repeated stimulation causes an accumulation of extracellular potassium ions and reduces the efficiency of membrane repolarisation (MacIntosh et al., 2012).

The M-wave has been commonly used as a marker for peripheral fatigue (Farina et al., 2004) and neuromuscular junction transmission failure (Bigland-Ritchie *et al*., 1982). This study shows that the majority of M-wave characteristics do not change as performance fatigability develops, either with PNS or NMES. The M-wave duration change seen with NMES may be relevant to this but can only be applied to NMES, not PNS, and is most likely caused by fatigue in superficial muscle fibres. Therefore, based on the findings of this study, there is a disassociation between sarcolemmal excitability and force generating capacity during stimulated contractions of the VL, with the latter largely explicable by decreased functionality of actin–myosin cross-bridges and abnormal Ca^{2+} handling (Cheng et al., 2018), and caution should be taken when using M-waves to normalise muscle activity.

Here we provide evidence to support the previously suggested theory that NMES recruits muscle fibres in a non-selective manner (Bickel et al., 2011). In the context of the majority of studies applying NMES over the muscle surface directly, it remains to be seen whether the same principle applies to stimulation applied over the nerve. However, irrespective of recruitment pattern, results from the present study suggest that the pool of muscle fibres available for recruitment using PNS is greater and potentially encapsulates a larger volume of muscle, rather than the superficial area targeted by NMES. With PNS, the lack of change in conduction time indicates that nerve function is not affected throughout the fatiguing protocol.

4.4 | Limitations and future work

The data herein demonstrate clear differences between electrical stimulation protocols with regards to myoelectrical measures of muscle and neuromuscular performance. Furthermore, it must be acknowledged that these data are from healthy, young participants and it is not clear if the same outcomes, including levels of tolerability, would be observed in older participants, in whom such interventions would be more applicable. As an additional limitation, participants were not asked to refrain from caffeine, alcohol, or other drugs before each session. Furthermore, voluntary activation has been implicated as a limiting factor of voluntary force output (Mileva et al., 2012). Although afferent feedback influences force generation during/following electrical stimulation, this appears to be at higher frequencies than those applied here (Collins et al., 2002). Additionally, as the M-wave represents sarcolemmal excitability, other aspects of performance fatigability such as reduced intramyocellular Ca^{2+} reuptake and sensitivity have not been directly quantified here and require further investigation (Cheng et al., 2018; Enoka & Duchateau, 2016). Given the evidence that chronic NMES applied directly over the muscle improves muscle function (Acaröz Candan et al., 2019) and attenuates muscle atrophy (Kern et al., 2014), while seemingly only activating a superficial area of muscle fibres, PNS over a similar time course may provide similar benefits, potentially with better acceptability. A longer-term protocol would require optimisation based on the responses of participants, and could provide further mechanistic insight, such as local and non-local muscle molecular and neural adaptations.

5 | CONCLUSION

This investigation found that level of whole muscle force reduction is not dependent on stimulation location. However, myoelectrical characteristics were found to change in response to NMES only, specifically M-wave duration and RD. We suggest that this difference provides evidence of a larger pool of muscle fibres being recruited when stimulating the motor nerve. Furthermore, PNS requires a lower intensity of stimulation to produce the same force and is more comfortable as a result of this. Collectively, these results suggest that PNS may be a more effective tool for rehabilitation than NMES. Future long-term interventions, particularly in clinically relevant populations, are warranted.

COMPETING INTERESTS

The authors have no conflict of interest to declare.

AUTHOR CONTRIBUTIONS

T.B.I., D.M., P.J.A., C.A.G., B.E.P. and M.P. conceived and designed study. T.B.I. and D.M. conducted experiments. T.B.I. analysed data and wrote the manuscript. All authors have read, contributed to and approved the final version of this manuscript and agree to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved. All persons designated as authors qualify for authorship, and all those who qualify for authorship are listed.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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