2016•2017
FACULTEIT GENEESKUNDE EN LEVENSWETENSCHAPPEN
master in de revalidatiewetenschappen en de kinesitherapie

Masterproef

Length-dependency of muscle fatigue and recovery in type I and type II mice muscle

Promotor :
dr. Pieter VAN NOTEN

Jasper Haesen , Toon Leysen
Scriptie ingediend tot het behalen van de graad van master in de revalidatiewetenschappen en de kinesitherapie

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The realization of this master thesis would not have been possible without the help of some other people. Therefore, we would like to thank our promotor, Dr. Pieter Van Noten, for his support throughout this process.

Furthermore, we want to thank the Hasselt University for all chances she give to all of her students and especially the faculty of rehabilitation sciences and physiotherapy for all support throughout our five-year education.

And last, but not least, a very special thanks to all of our friends and family for supporting us, even at most difficult moments.

Millerweg 75 3700 Sluizen, 06/06/2017 **J.H.**

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Research context

Muscle fatigue is a well-known and typical characteristic in skeletal muscle. Where in healthy subjects, fatigue is associated with normal reaction due to repetitive and intensive activation, it is considered as symptom in a state of disease like multiple sclerosis (MS). Since fatigue is dependent on many different environmental (humidity, warmth, intensity) and human body associated factors, it is important to create a good experimental setup to evaluate the impact of fatigue in disease models.

This study was labelled as a pilot study, to test the newly developed setup in function of muscle fatigue. At REVAL (study centre of the Hasselt University), research is going on with focus on MS. Knowing this new setting worked, means a start to future research. Next, in testing the validation and reliability of the setup, a pilot study was performed. In this pilot study, three major muscle output variables (stimulation frequency, muscle length and fatigue) were evaluated. As this pilot study was used to explore the setup, a small sample size of mice, yet with large inter-individual differences (in weight, age, species) was used.

The research protocol was delivered by our promotor Dr. Pieter Van Noten, just like setup and dissected samples. The protocol was performed by Toon Leysen and Jasper Haesen, both student of the Hasselt university. This research was conducted in terms of the master's degree program of physical therapy and rehabilitation sciences. With aim of proving being capable to perform research, resulting in information which could be used in evidence-based practice. Initially, another topic was chosen, but due to extern factors this study was not feasible. So, only at the end of April could be started with current topic. All had to be completed in six weeks. After acquisition, data-analysis and processing data were performed. Results and interpretation of this information were used to write this manuscript. The purpose was to evaluate the relation force-frequency and force-length, by drawing both a force-frequency and force-length relationship, also results and possible significant differences were calculated. Data of force-length relationship showed deviating values by possible error in analysing-software (Labview). Hence, a representative result could not be obtained. Main part of the study contained testing fatigability of the muscles and possible length-dependency. All tasks were completed by both students together, with supervision and help of Dr. Pieter Van Noten.

1. Abstract

Background: Muscle fatigue is a well-known phenomenon. Knowledge about possible affecting factors (length, fibre type, stimulation frequency, …) is necessary before applying it to specific circumstances, like MS.

Objective: To assess the influence of length on peripheral muscle fatigue and recovery.

Methods: Soleus (SOL) and Extensor Digitorum Longus (EDL) muscles were dissected out of 14 mice. The optimal length (L_0) was set as maximal isometric active (total – passive) force production by supramaximal electric stimulations. Force output was evaluated relative to muscle cross-sectional area (CSA) by one twitch (1Hz, 60s rest) and one tetanic (350ms train, 120s rest) contraction at 50Hz for SOL, 100 Hz for EDL. Tetanic contractions at different stimulation frequencies (10, 25, 50, 80, 100, 125 and 150 Hz) evaluated for the force-frequency relation. Afterwards standard tetanic contractions (50Hz for SOL, 100Hz for EDL, 350ms train, 120s rest) at different muscle lengths (L_0, $L_{0\text{-}1mm}$, L_{0+1mm}, $L_{0\text{-}2mm}$, L_{0+2mm}, $L_{0\text{-}3mm}$ and $L_{0\text{-}4mm}$) were used to evaluate the force-length relation. Finally, fatigue (active and passive force) at random muscle was assessed by frequently repeated (rest interval 2.5s for SOL, 20s for EDL) standard tetanic contractions for 10 minutes after which two more tetanic contractions followed (5 and 10 minutes after the end of the fatiguing protocol) to evaluate recovery from fatigue. Data were analysed by Oneway analysis of variance (non-parametric) and matched pairs Wilcoxon on JMP Pro 12. Significance level was set on $p<0.05$.

Results: EDL and SOL reached maximal active force upon stimulation at >150 and 100 Hz, respectively, not at used stimulation frequencies (100 and 50Hz). Both muscles show a similar fatigue-process at optimal length (L_0) with a decline in force between the start (FATstart) and the end of the 10 minutes fatiguing protocol (FAT10) ($p<0.05$), although EDL fatigued less than SOL. They both recover till the initial level, SOL force recovered above the initial force level. Length-dependency present in limited number of lengths for SOL (L_0 and $L_{0\text{-}3mm}$) and absent for EDL. Passive force at L_0 did decline significantly between FATstart and FAT10 for EDL ($p<0.05$) but not for SOL. EDL at L_0 recovered till the initial passive force level, but SOL did not. Length-dependency for passive force changes occurred for SOL (L_0 and L_{0+2mm}), but not for EDL.

Conclusion: Newly developed setup worked in expected way. Limited link between muscle length and muscle fatigue. Possible reasons are the small sample size and the variability between mice. Further, standardized research is necessary.

2. Introduction

Fatigue is defined as a reversible decline in muscle performance associated with muscle activity, so fatigue can be classified as a normal physiological process. Although, it can be associated with different pathologies and is characterized by a decline in force, a decrease in contraction velocity and an increase of relaxation time (Allen, Lamb, & Westerblad, 2008; Fitts, 1994). Literature differentiates central and peripheral fatigue. The location where they act upon, is the most important difference between these two. Generally, voluntary muscle activity is elicited in the brain. The electrical impulse from the brain cells is directed from the nervous system towards the muscles. At this neuromuscular junction, the electrical impulse is transferred from the nervous membrane towards muscle fibre membrane by a chemical synapse (Burgerhout et al., 2006). The neuromuscular junction, therefore bridges the communication between the central nervous system and the peripheral muscle. So, in case that fatigue originates in the nervous tissue, before the impulse reaches the neuromuscular junction, is defined as central fatigue. Fatigue onset peripheral from the junction, is associated with the muscle and thus peripheral fatigue (Bigland-Ritchie, Jones, Hosking, & Edwards, 1978). Also, central fatigue is an activity-induced inability to activate a muscle voluntary, while peripheral fatigue points out an inability of the muscle itself to deliver force (Enoka & Stuart, 1992; Nordlund, Thorstensson, & Cresswell, 2004). In that way, central fatigue can be categorized as an neural problem, and not just a muscular problem.

In this study, focus will be on intrinsic muscle fatigue, peripheral to the neuromuscular junction. This kind of fatigue has an influence on the crossbridge-cycle, a cyclic interaction between myofilaments actin and myosin. This cycle starts after an electrical impulse depolarized the muscle fibre membrane, causing release of calcium (Ca^{2+}) by sarcoplasmic reticulum (SR). Released Ca^{2+} binds to Troponin-C (TnC), which results in ability of myosin to bind actin, forming a crossbridge. Adenosine triphosphate (ATP) is necessary to complete this binding and to produce force. During ATP hydrolysis, ATP splits in adenosine diphosphate (ADP) and inorganic phosphate (Pi), this Pi-release is thought to start the transition in actomyosin binding from a weakly bound low-force state to a strongly bound high-force state, followed by a powerstroke, so the muscle is able to deliver force (Ebashi, Ebashi, & Kodama, 1967; Huxley, 1957; Metzger & Moss, 1990). (See Fig. 1)

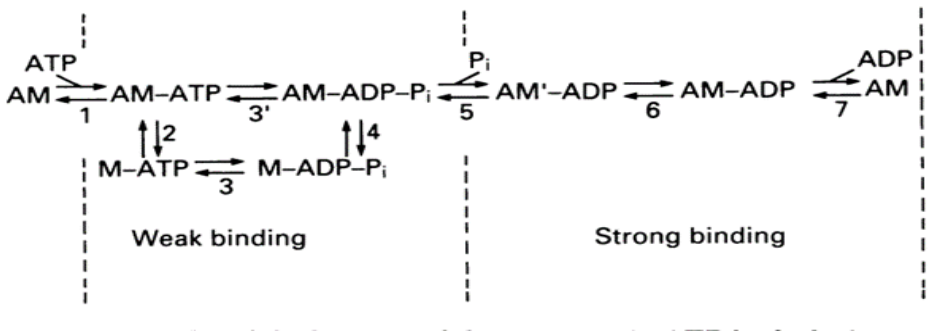

Fig. 1: Schematic model of actomyosin ATP hydrolysis reaction during skeletal muscle contraction (A: Actin, M: Myosin). (Metzger & Moss, 1990)

Muscle fatigue is a process and three phases are distinguished as result of repeated contractions (Fig. 2). Phase one consists of an initial fast decline of tetanic force (-10-20%) in combination of an increased amount of free myoplasmic Ca^{2+} concentration ($[Ca^{2+}]_i$), which reflects a reduction in crossbridge force-generating capacity. In contrast, phase two has a relatively long duration and shows a slow decline in both force and tetanic $[Ca^{2+}]_i$. At last, phase three shows a rapid decline of both tetanic force and tetanic $[Ca^{2+}]_i$, which is the result of a decreased myofibrillar Ca^{2+} sensitivity, so less Ca^{2+} can be bound to Troponin-C (TnC) and less crossbridges can be formed (Lannergren & Westerblad, 1991; Westerblad & Allen, 1991).

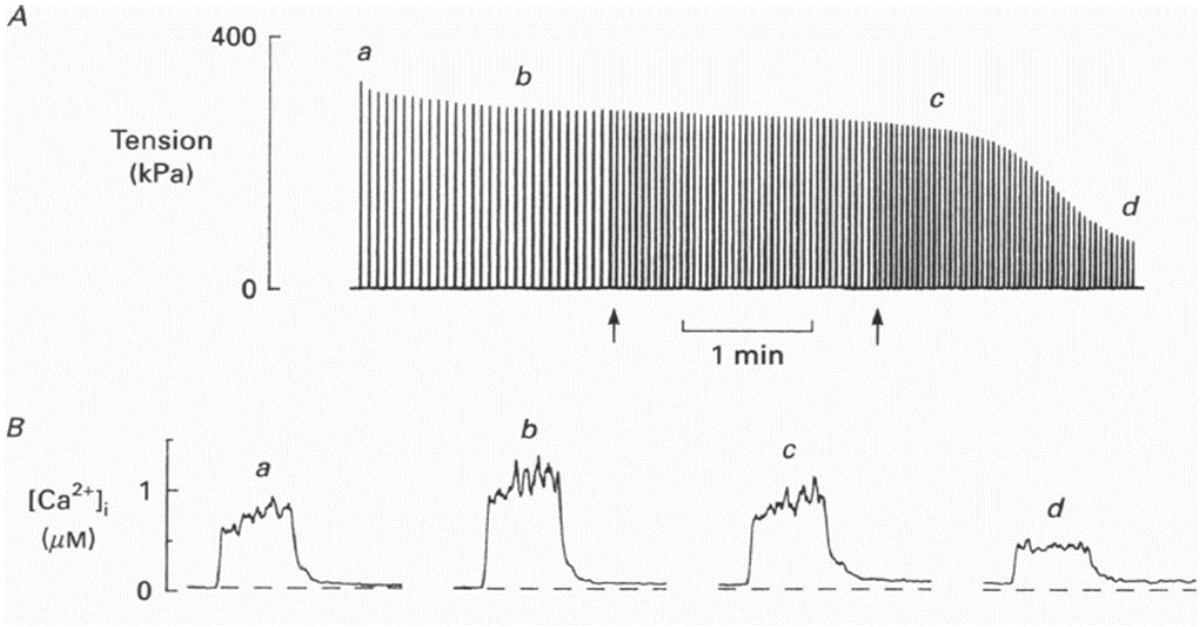

Fig. 2: A, Continuous force record in which different phases of force output showed up. B, $[Ca^{2+}]_i$ – record during repeated tetani. From Westerblad and Allen (1993).

Many underlying mechanisms of this altered release of Ca^{2+} by the sarcoplasmic reticulum (SR) and reduced myofibrillar sensitivity to Ca^{2+} have been reported. Increased production of Pi and reactive oxygen species (ROS) are just two examples. Factors like lactate, pH and H+ were already mentioned in literature as possible causes, but research showed only little effect on muscle fatigue. Because differences were not representative to the amount of fatigue (Cady, Jones, Lynn, & Newham, 1989; Usher-Smith, Fraser, Bailey, Griffin, & Huang, 2006). The increase of Pi results from the breakdown of ATP and phosphocreatine during the powerstroke of the crossbridge cycle. Increased production of Pi results in an inhibition of the crossbridge-cycle and SR Ca^{2+}-pumps, due to a decreased free energy change of ATP hydrolysis, resulting in an impaired reuptake of Ca^{2+} (Dahlstedt, Katz, & Westerblad, 2001; Dawson, Gadian, & Wilkie, 1980; Duke & Steele, 2001). Another consequence of this increased Pi production is a decrease in Ca^{2+}-content in phase three of the protocol. This by the fact that inside the lumen of SR, Pi binds to Ca^{2+}, which results in a decrease in free, releasable Ca^{2+}, so less can bind to TnC and less crossbridges can be formed (Fryer, Owen, Lamb, & Stephenson, 1995; Westerblad & Allen, 1996). Another fact, literature showed a difference in Pi production in both fast and slow twitch muscle fibres. In case of fast twitch fibres (Type IIb), a higher amount of Pi is present than in slow twitch fibre (Type I) after muscle contraction. A possible explanation was mentioned by Bottinelli, Schiaffino, and Reggiani (1991), they concluded that each muscle fibre type contain a specific isozyme of the contractile protein myosin, the protein containing the adenosine-triphosphatase (ATPase), which delivers ATP and in that way Pi during contraction. Fast twitch fibres contain a higher myofibrillar ATPase activity compared to slow twitch fibres. So, if an increased production of Pi results in increased fatigue, the latter confirmed why fast twitch fibres have higher fatigability than slow twitch fibres (Schiaffino, Hanzlikova, & Pierobon, 1970). ROS, which are continuously generated inside cells as by-products of oxidative metabolism (Chance, Sies, & Boveris, 1979), has an influence on the rate of fatigue at 37°C, but not at 22 °C (Andrade, Reid, Allen, & Westerblad, 1998; He et al., 2016), and is suggested to reduce myofibrillar sensitivity to Ca^{2+} (Moopanar & Allen, 2005), SR Ca^{2+} release (Bruton et al., 2008) and membrane excitability (McKenna et al., 2006). Summarizing literature, the force decline in phase one of the fatiguing protocol seems related to a decreased force per individual crossbridge, whereas in phase three during the fatiguing protocol, a decrease in number of active force-generating crossbridges is suggested (Nocella et al., 2011).

Consequently, if fatigue results from metabolic events associated with crossbridge interactions, then fatigue should be related to the amount of actin and myosin overlap, as it determines the amount of cycling crossbridges. Therefore, greater fatigue should be observed at optimal length because actomyosin overlap is optimal and thus the amount of cycling crossbridges is maximal. Fitch and McComas (1985) were the first to support this theory when comparing in vivo human ankle dorsiflexor muscles at optimal length with shortened length. In their study ankle dorsiflexors were stimulated at their optimum length (15° plantar flexion) and at their fully shortened length (25° dorsiflexion) by indirect tetanic contractions at 20 Hz for 90 seconds. A significant reduction in twitch and tetanic torque was found at the optimum muscle length, but not at shortened muscle length. Also, Lee et al. (2007) who tested in vivo human quadriceps in short and optimal muscle lengths (15° and 90° of knee flexion respectively) observed a greater absolute and relative fatigue at optimal muscle length. MacNaughton and MacIntosh (2006) were critical about the traditional way of active force production calculation: subtraction of the pre-contractile passive force from the total force. They discuss that passive force measured before contraction is not related to the passive force during contraction as in whole muscle preparations fascicle length shortens upon activation. A better calculation can be made by subtracting the passive force that was associated with the fascicle length reached at the peak of the contraction. Theoretical background about this alternative calculation could be found in the generalized Hill model that consist of a passive element in the upper branch, which is connected to a lower active branch in parallel (Hill, 1970). The total stretch during contraction is multiplicatively split into the elastic stretch and the active stretch (Goktepe, Menzel, & Kuhl, 2014). MacNaughton and MacIntosh (2006) reported with their alternative calculation method in an in vitro study from isolated mice medial gastrocnemius muscles the relative fatigue is greater at short length and the absolute fatigue is greater at long length. MacIntosh (2017) later called this finding 'the length dependence of fatigue' and explained the underlying mechanisms of greater relative fatigue at short muscle length and absolute fatigue at long length by the term 'length dependence of activation' (LDA). LDA is based on the assumption that the shift of optimal overlap is due to increased Ca^{2+} sensitivity at longer lengths, because at longer lengths myofilaments are closer together allowing greater force than expected.

Summarized, literature is undefined about the length dependency of fatigue. Furthermore different measurement protocols were used and made interpretation of underlying mechanisms difficult. This study focusses on the impact of muscle length on peripheral fatigue in isolated isometric whole-muscle (SOL and EDL) contractions.

3. Methods

This section was based on earlier research by Derave, Op 't Eijnde, Ramaekers, and Hespel (2005).

3.1. Animals

14 mice were maintained under conventional breeding conditions with food and water ad libitum and were kept on a 12:12 h light:dark cycle. The experimental protocol was approved by the Ethics Committee for Animal Research at the Hasselt university.

3.2. Muscle contractile properties

At the start of the study, mice were anaesthetized by an intraperitoneal injection of pentobarbitone sodium (Dolethal 150mg/kg b.w.). After the protocol, mice were killed by cervical dislocation or an overdose of Dolethal (up to 200mg/kg b.w.). SOL and EDL from both legs were dissected and incubated in organ baths containing Krebs-Henseleit solution (118 mmol l^{-1} NaCl, 25 mmol lK1 NaHCO3, 5 mmol lK1 KCL, 1 mmol lK1 MgSO4, 1 mmol lK1 KH2PO4, 2.5 mmol lK1 CaCl2 and 1 mmol lK1 glucose), which were continuously gassed with a mixture of 95% O_2 and 5% CO_2 and maintained at 25°C. During dissection, wires were attached to the tendons of SOL and EDL to allow vertical mounting in the incubation batch with one tendon attached to a force transducer and the other to a fixed point. After 10-15 minutes of rest in the incubation buffer, muscles were stimulated by platinum electrodes flanking the muscle, which delivered capacitor discharges (pulse duration 1ms; 20V). Except when indicated, all tetanic contractions were set at 350 ms of train stimulation with two minutes rest interval at 50 and 100 Hz stimulation frequency for SOL and EDL, respectively. Maximal active force production (total force – passive force) was found by tetanic stimulations and small length adjustments at the initial rise of the passive force (± 5 mN) and was set as reference optimal muscle length (measured as L_0). First, in order to evaluate muscle force output (absolute and relative to cross-sectional area), SOL and EDL muscles were electrically stimulated by a twitch (1Hz, 60s rest) and tetanic contraction at L_0. A force-frequency relationship was established by six tetani with increasing stimulation frequency of 10, 25, 50, 80, 100, 125 Hz for SOL and 25, 50, 80, 100, 125, 150 Hz for EDL. Thereafter, a force-length relationship was evaluated with seven tetani at different muscle lengths (L_0, $L_{0\text{-}1mm}$, L_{0+1mm}, $L_{0\text{-}2mm}$, L_{0+2mm}, $L_{0\text{-}3mm}$ and $L_{0\text{-}$

$_{4mm}$). At last, fatigability was evaluated by a 10 minutes repetitive tetanic stimulation protocol with 350ms contractions separated by 2.5 seconds rest interval for SOL and 20 seconds for EDL. Force output (active, total and passive) is sampled every minute of this 10min protocol and is labelled as FAT1 till FAT10, with FATstart as the initial reference force (100%) at the start of the fatigue-protocol. Recovery after 5 and 10 minutes was sampled by a single tetanus (RECOV5 and RECOV10) following cessation of the fatigue protocol. After completion of this protocol, muscles were weighted and measured.

This protocol has a total duration of 53 minutes in which two muscles are tested at the same time. In total, sample-size contains 26 SOL and 20 EDL muscles. Schematic presentation of the study outline is showed in Appendix Fig. A.

3.3. Outcome measures

Primary outcome measure in this study was force, expressed in Newton (N). Muscle length and weight were used to calculate muscle CSA, estimated by dividing wet muscle mass by the product of optimal muscle length and 1.06 g/cm³. So, muscle force could be expressed relatively in N/cm². Both passive (F_p) and total (F_t) force were measured, active force (F_a) were calculated by subtracting the passive from the total force ($F_a = F_t - F_p$). F_p were measured before stimulation. Another variable measured is the highest slope of the ascending ($\Delta F/\Delta t$) and lowest slope of the descending ($-\Delta F/\Delta t$) limb of every contraction, which means maximal and minimal rate of force development and relaxation, respectively. Muscle fatigue was evaluated by a possible decline of F_a and $_{Fp}$, followed by the degree of recovery. Active and passive forces were expressed relatively, to the initial force at the start of the fatiguing protocol (FATstart) to create a representative image about the different variables.

3.4. Statistics

Statistically, testing results parametrically was not allowed, because of small sample size (<30). So, non-parametric tests were used. Differences in relative twitch and tetanic contractions from SOL and EDL were analysed by one-way analysis of variance in a non-parametric way, so Wilcoxon/Kruskall wallis test was used. To analyse different stimulation frequencies and muscle lengths, matched pairs (Wilcoxon) were used. To evaluate the effect of muscle length on fatigue and recovery, also matched pairs (Wilcoxon) were used, these pairs were formed by a combination of two different test moments. When these matched pairs showed a

significant difference in force, all possible combinations of different muscle lengths were made to control if some significant difference between certain muscle lengths could be found. A non-parametric comparison for each pair using Wilcoxon method was used (Portney & Walkins, 2009). All statistics were performed using JMP Pro 12 software. A-Significance level was set at ≤0.05. All data are shown as mean ± 1 Standard Deviation.

4. Results

Force output. Mean weight of samples was 9.82 ± 1.56 mg and 13.7 ± 1.7 mg for SOL and EDL respectively. Mean length was 12.2 ± 0.86 mm for SOL and 13.6 ± 1.37 mm for EDL. Mean twitch force/CSA for SOL and EDL was 3.466 ± 0.78 and 4.051 ± 1.32 N/cm² respectively, no significant difference was found between them ($p = 0.1987$). Mean tetanic force/CSA for SOL and EDL was 22.347 ± 4.63 and 31.026 ± 6.9 N/cm² respectively, a significant difference was found here ($p < 0.001$) (Fig. 3). Results for tetanic force, EDL muscles produced higher force per CSA compared to SOL muscles.

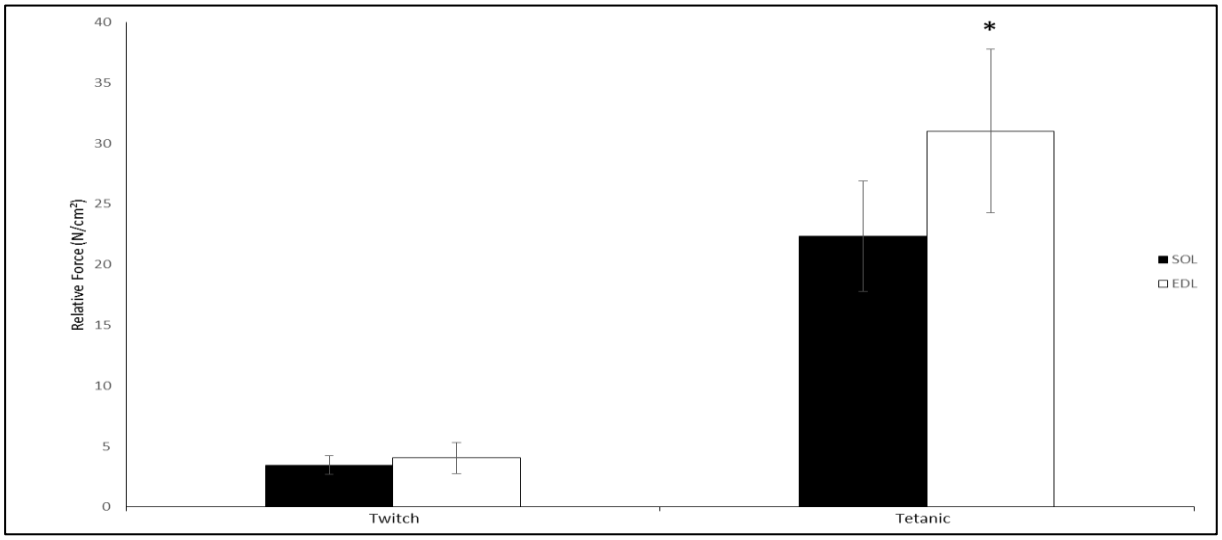

*Fig. 3: Relative (per CSA) Twitch and Tetanic force, expressed in N/cm². SOL: soleus, EDL: extensor digitorum longus.*Significant difference between muscles, p<0.05.*

Force frequency. Comparing SOL and EDL at different stimulation-frequencies (25, 50, 80, 100 and 125 Hz) showed a difference in absolute force output ($p < 0.05$) at each frequency. Matched pairs analysis between different frequencies, in function of the force-frequency relationship (see Fig. 4), showed differences till 100Hz for SOL in case of relative and absolute force output ($p < 0.05$). Yet no difference is found between 100 Hz and 125Hz for relative ($p = 0.0830$) and absolute ($p = 0.2412$) force output. For EDL, relative and absolute force output were different for all tested frequencies (25, 50, 80, 100, 125 and 150Hz). Schematic presentation of relative force of both SOL and EDL showed in Appendix Fig. B.

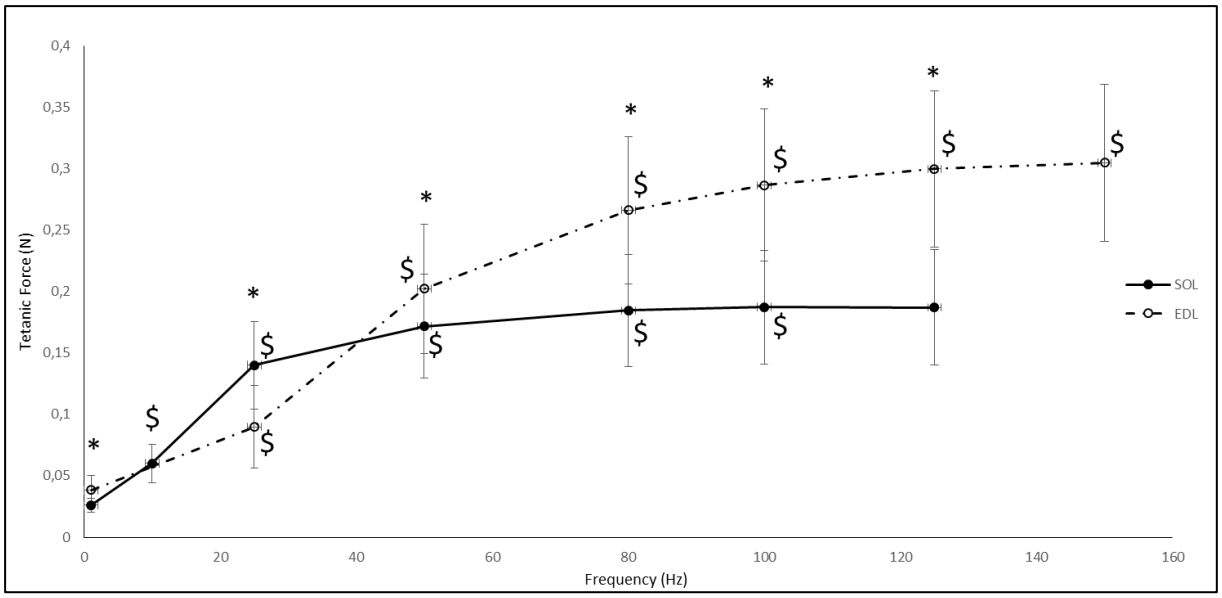

*Fig. 4:: Force - Frequency Relationship for both SOL (filled circles) and EDL (open circles). Expressed in Absolute active Force (N). *Significant difference between SOL and EDL at present frequency. $ significant difference relative to previous frequency. p<0.05.*

Length-dependency of fatigue and recovery. Before length-dependency of fatigue can be checked, it is necessary to evaluate if the muscle is able to fatigue in a normal way and if they fully recover. If muscle force does not recover to the initial force output, muscle damage might be induced present. All SOL and EDL muscles, measured on the optimal length (L_0), were screened if they actually fatigue and recover. SOL and EDL force output declined during the 10 minute fatiguing protocol (SOL: p=0.0007, EDL: p=0.0121) and made full recovery (comparing RECOV10 to FATstart). Moreover SOL active force at RECOV10 was well above FATstart (p=0.0108). For EDL, no difference was found between FATstart and RECOV10 (p=0.3745).

The general occurrence of fatigue in muscles at L_0 was tested by comparing each test moment with FATstart. All test moments differed from FATstart ($p<0.05$). Also, a comparison of force between every minute of the fatigue-protocol was made. As mentioned above, a decline in force was shown between FAT start and FAT 10 for SOL. In both SOL and EDL, force decline happened in the first part of the protocol (till FAT6), afterwards no differences were found. Results are shown in Fig. 5. At each test moment, a difference showed up between SOL and EDL.

To test the actual length-dependency of fatigue: when looked for a difference between FATstart and FAT10 for each length individually, a significant decline took place for every length, except $L_{0\text{-}3}$ and $L_{0\text{-}4}$ in SOL muscles and only for $L_{0\text{-}2}$, $L_{0\text{-}1}$ and L_0 in case of EDL (see Fig. 6). All possible combinations in length and time of fatigue protocol were tested. Not many differences were shown, especially for EDL, where no significant differences showed up. In case of SOL, the comparison between length L_0 and $L_{0\text{-}3}$ showed a significant difference at most of the test moments (FAT 1, 2, 3, 6, 7, 8, 9 and 10). After 10 minutes, L_0 declined to 57% of the initial force, while $L_{0\text{-}3}$ only declined to 91% (see Appendix Fig. C for a schematic comparison between both). Comparing other lengths, no remarkable difference could be found.

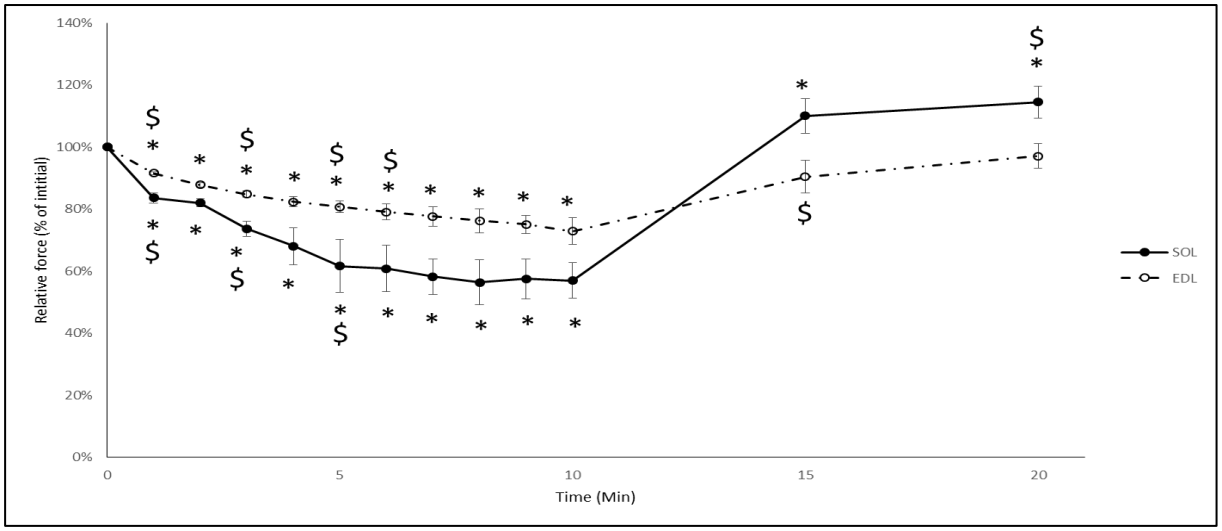

*Fig. 5: Fatigability of SOL (filled circles) and EDL (open circles), Tetanic force (relative to initial force) during 10min of repeated fatiguing stimulation and 10min of recovery.. *Significant difference relative to FATstart. $ Significant difference compared to previous test moment. p<0.05.*

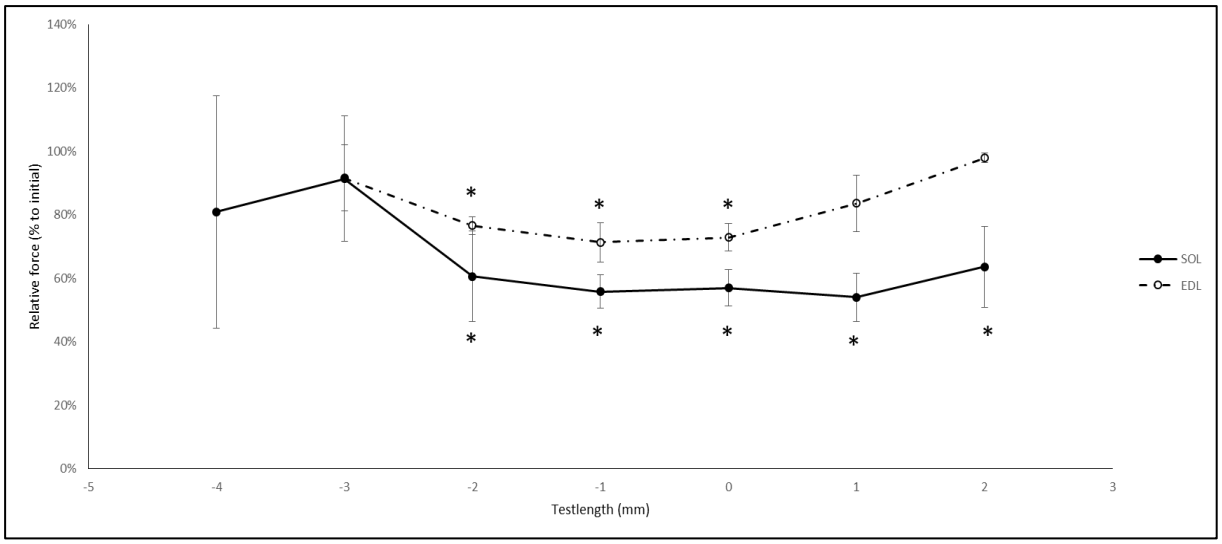

*Fig. 6: SOL and EDL: FATstart - FAT10: Influence of length to relative level of fatigue after 10 minutes of stimulation. *Significant difference between FATstart and FAT10 per test-length. p<0.05.*

Length dependency of passive force: Changes during fatigue and recovery. Passive force variation during fatigue was assessed similar to active force. First, the effect of fatigue at L_0 was evaluated, later, the length dependency of fatigue was evaluated. Relative passive force changes (passive force at FAT start= 100%) for SOL and EDL muscles by L_0 were analysed separately. For fatigue (FAT start – FAT10) no significant difference was found for SOL (p=0.0900) but a significant decline was found for EDL (p=0.0367). Recovery (FATstart – RECOV10) was significantly different for SOL (p= 0.0009), which means there was a significant decline and passive force for SOL did not recover to the initial 100%. This was not the case for EDL, where no significant difference was found (p= 0.1092).

Comparing passive force from all consecutively minutes of the fatigue-protocol between FATstart and FAT10. For SOL significant differences were found between FATstart-FAT1, FAT1 –FAT2, FAT3-FAT4 and by FAT9-FAT10 ($p<0.05$). For EDL, no significant changes between consecutive minutes were found ($p>0.05$). (see Fig. 7)

Comparing all test moments with FATstart. For SOL significant differences were found at FAT1, FAT2, FAT3 and RECOV5. For EDL significant differences were found at FAT3, FAT4, FAT5, FAT6, FAT7, FAT8, FAT10, RECOV5 and RECOV10. (see Fig. 7). Comparing passive force changes between SOL and EDL at L_0. Significant differences were found for FAT1, FAT2, FAT3, FAT4, FAT5, FAT8, FAT10 and RECOV5.

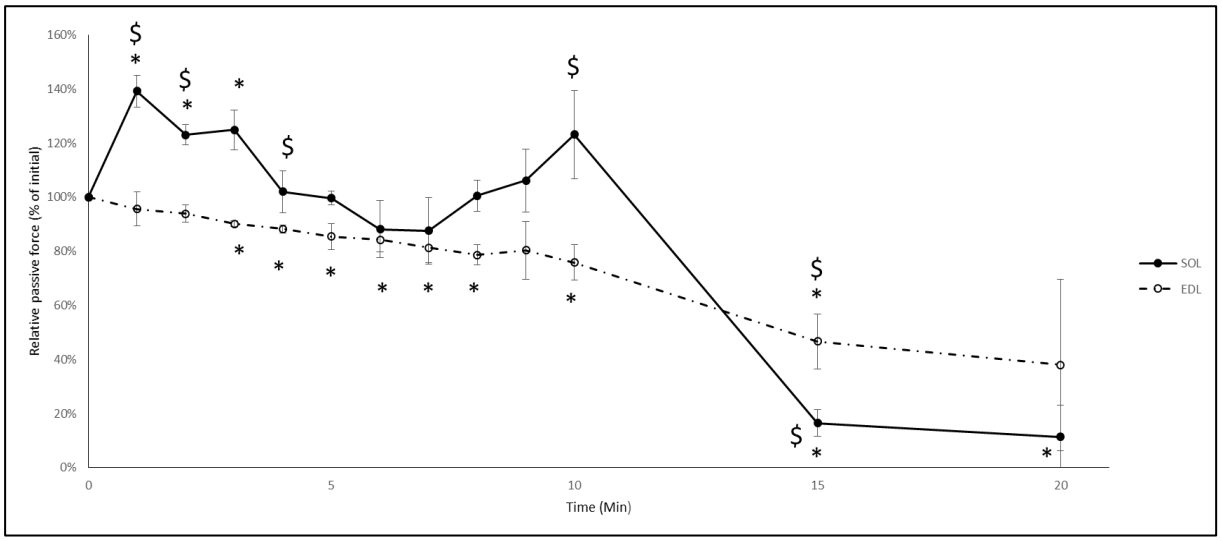

Fig. 7: Passive force (relative to initial force), 10min of repeated fatiguing stimulation, followed by 10min rest interval. SOL: soleus, EDL: extensor digitorum longus. Significant difference relative to FATstart. $ Significant difference compared to previous test moment. p<0.05.*

The length-dependency of passive force changes during fatigue and recovery were tested by all combinations of protocol lengths with their passive force at every test moment. For EDL, no significant differences showed up. For SOL the comparison of length L_0 and L_{0+2} showed a significant difference at FAT1 , FAT2 and RECOV5. The comparison between L_{0+1} and L_{0-1} showed a significant difference at FAT8 and FAT9. At FAT1 also L_{0+2} differed significantly with L_{0-1} and L_{0+2} with L_{0+1}. At FAT5 L0 differed significantly with L_{0-1}. At FAT7 L_0 differed significantly with L_{0-2}. And at FAT 8 there were significant differences between L_{0-1} with L_{0-2} and L_0 and L_{0+1}.

5. Discussion

Results showed a difference in force-frequency relationship in SOL and EDL, a decline in muscle force during the first part of the fatigue protocol and limited length-dependency of muscle fatigue. Only EDL passive force declined significantly after 10 minutes of fatigue protocol.

In this study, first of all, as this was a pilot study, mice were donated from other studies. Large variations in age, strain, gender and weight were present. Age differences are known to decrease absolute maximal force, however peak force (normalized to muscle CSA) for both SOL and EDL is not age dependent (Graber, Kim, Grange, McLoon, & Thompson, 2015). So by looking at the twitch and tetanic force (N/cm^2) per CSA, insight was given about the contractile properties independent of age. The effect of weight and length on force output could not be tested. Mean relative twitch force for SOL (50Hz) and EDL (100Hz) was 3.466 ± 0.78 and 4.051 ± 1.32 N/cm^2 respectively. When compared to other studies, Tallis, James, Cox, and Duncan (2012) found relative twitch force for SOL (stimulation frequency: 70Hz) and EDL (150Hz) was 3.27 ± 0.26 and 6.62 ± 0.26 N/cm^2 respectively, for 8-10 weeks female white mice. Mean relative tetanic force for SOL and EDL was 22.347 ± 4.63 and 31.026 ± 6.9 N/cm^2 respectively. Derave et al. (2005) illustrated a tetanic stress at 100Hz 350ms duration for SAMR1 mice 60 weeks old for SOL and EDL, 21.6 ± 1.7 and 36.9 ± 3.8 N/cm^2 respectively, while James, Altringham, and Goldspink (1995) showed a smaller relative tetanic force for EDL (23.3 ± 0.77 N/cm^2). Absolute tetanic force in current study was 171.08 ± 44.44 mN and 293.89 ± 65.41 mN for SOL and EDL respectively. Compared to Derave et al. (2005), absolute tetanic force was 210 ± 7 and 355 ± 19 for SOL and EDL respectively. So, despite a widespread standard deviation was visible, both SOL and EDL in vitro performance properties were similar in relative and absolute force to earlier research. According to Tallis et al. (2012), these present differences could possibly be attributed to muscle-fibre type differences due to variation in strain and age of mice. In that way, the large variability of included mice is a weakness of present study and might be related to the large spread in results. After testing 26 SOL and 20 EDL muscles, the fact is the newly developed setup worked and showed relevant results in this research.

Both SOL and EDL showed a clear relation to stimulation frequency. Maximal force output is registered at 100Hz for SOL. EDL muscle will possibly not have a maximal force production at 125Hz, because of the significant difference between 125 and 150Hz. Force-frequency relation could be drawn, which showed a steep ascent ending on a plateau. So, higher frequency results in higher force due to higher release Ca_{2+} by depolarization of the muscle fibre membrane. Like Derave et al. (2005), in this study SOL and EDL were stimulated at 50 and 100Hz respectively, so a submaximal stimulation was performed for both muscles. More specific for SOL the relative difference between 50Hz – 100Hz was 7.7% and for EDL the relative mean difference between 100Hz – 150Hz (suspicious maximally stimulation frequency) was 6.4 %. This indicated a nearly maximally stimulation for both muscles. which is important because Lee et al. (2007) showed a significant higher fatigue at low frequency (14.3Hz) compared to high-frequency (60Hz), also known as low-frequency fatigue. These values are similar to frequencies used by Edwards, Hill, Jones, and Merton (1977). They concluded low-frequency fatigue happened with a stimulation-frequency of 20Hz and labelled 80Hz as high-frequency stimulation.

So, to link results to the used frequency in function of the fatigue protocol, low-frequency has to be taken into account. A suggestive explanation was already given in literature by the fact that in low-frequency stimulation less Ca^{2+} will be released from SR and will result in a decline in force. Although, action potential is normal at this frequency, so another explanation has to be found (Westerblad, Duty, & Allen, 1993). Literature concluded a decreased Ca^{2+} transient at this level of stimulation, a Ca^{2+} transient along the steep ascending portion of the force-calcium concentration curve. So a decline in Ca^{2+}-release will result in a large decline in force, while in high-frequency stimulation Ca^{2+}-transients is on the asymptotic plateau of the force-calcium concentration curve and a decline Ca^{2+}-release will result in a small change of force (Chin & Allen, 1996). However, even if the used frequency did not stimulate both muscle-types maximally, decline in force cannot be due to low-frequency fatigue.

Both SOL and EDL muscles showed a decline in muscle force output during the fatigue protocol. Generally a significant decline in tetanic force each minute with a plateau reaching the end at 10 minutes. Also recovery till 100% (initial tetanic force) is matched, only SOL increases above initial force. So, the SOL muscles became stronger compared to the start at L_0. The difference between SOL and EDL in terms of recovery in the present study could not

be declared, but the degree of fatigue at the end of the 10-minutes fatigue-protocol was less high in EDL compared to SOL following the protocol, most important possible reason is the different rest-interval (2.5 sec for SOL, 20 sec for EDL). Though a difference was found at each test moment, a comparison between these two muscles cannot be made, as a result of the different protocols which were used. Derave et al. (2005) tested fatigability by six consecutive 2-minute bouts of repeated tetani (350ms) with decreasing rest-intervals (3.8, 3.1, 2.6, 2.1, 1.6, 1.3s) for both SOL and EDL. Resulting in higher degree of fatigue for EDL (remaining 10% of initial force) than for SOL (40%). Both SOL (70%) and EDL (35%) did not recover fully. So, intensity of the fatigue protocol determines the degree of both fatigue and recovery. In current study, intensity was possibly not high enough to show similar results.

The length-dependency of fatigue did show less significant differences than would be expected, possibly due to a small sample size. Because of the insufficient amount of samples, it was not allowed to use parametric tests. In non-parametric tests only SOL showed significant differences between several lengths, no length-dependency was found for EDL. An explanation could be found in lower degree of fatigue in EDL muscles. The purpose of this study was to look for a difference in fatigability between several lengths. Like mentioned earlier, only between L_0 and L_{0-3} for SOL, most of the time a significant difference was present, in which fatigue was less high in L_{0-3}. This result was also observed by Fitch and McComas (1985), they concluded higher fatigue occurred at longer lengths, because of the fact fewer crossbridge interactions occur at short length compared to the optimal length. Fewer crossbridges means less ATP-consumption and less fatigue. According to Fitch and McComas (1985), more fatigue on longer lengths results from a greater metabolic cost to activation, which could impair in excitation-contraction coupling at the level of the Ca_{2+}-release channel. For SOL this was demonstrated comparing FATstart to FAT10 for each length. Shortest lengths (L_{0-3}, L_{0-4}) did not show any difference, so did not fatigue. EDL showed divergent results, because no difference at lengths longer than the optimal length (L_{0+1}, L_{0+2}) were present. Possibly by lower degree of fatigue due to a different protocol.

Focussing on Fig. 7 the relative passive force for SOL seemed to increase comparing FATstart with FAT10, but the difference was not statistically significant. However comparing FAT9 with FAT10, there was a significant difference. A possible explanation can be found in a large standard error between FATstart and FAT10 ($p= 0.0450$). In contrast relative passive force for

EDL declined significantly comparing FATstart with FAT10. The difference between EDL and SOL was significant (p=0.0102), but it was not possible to compare these outcomes only by muscle type, because they had a different fatigue protocol. So, possible differences would not be representative. MacNaughton and MacIntosh (2006) mentioned that a significant decrease in passive force from dissected medial gastrocnemius of rats occurred from 50Hz fatiguing contractions. However Rassier, Lee, and Herzog (2005) resulted strong evidence for an increase in passive force from frog lumbrical muscles that is mediated by a length-dependent combination of stretch and activation. This could be explained by the molecular spring titin. This protein is the main contributor to passive force and the I-band of titin makes a connection between actin-filament at the Z-line and myosin at the A-band (Powers et al., 2014). Titin stiffness increased by increasing Ca^{2+} concentration (Leonard & Herzog, 2010; Powers et al., 2014; Rassier et al., 2005). Labeit et al. (2003) found the tetanic [Ca^{2+}] from the flexor digitorum brevis muscle (fast- twitch), for a given tension was generally higher during fatiguing stimulation than under control conditions. An increase in passive force after fatigue could be expected due to the spring filament titin. Another possible explanation, due to impaired reuptake of Ca^{2+} during fatigue, released Ca^{2+} could stay bound to Tnc (Dawson et al., 1980; Ebashi et al., 1967; Huxley, 1957). In that way, crossbridges could stay active and resulting in increasing passive force due to fatigue. Summarized there is contradiction between literature and several explanations are possible. Further research needs to be done to explain our results. This further research should contain passive force changes in fatigued muscles and should focus on muscle types and grades of fatigue.

Strengths of this study were the use of a newly developed protocol, with a combination of fatiguing the muscle and possible influence of length-dependency. Also limited need for external factors made it more easy to perform and to control all factors. Despite some limitations (see further), a lot of progression is possible, while results were already promising.

Limitations of this study were mainly the available sample size, 26 SOL and 20 EDL muscles. All muscles were randomly divided into seven different muscle lengths during the fatigue protocol. So, all different groups contained a maximum of four samples, which was too small to obtain representative results. Another important limitation was the variability between mice, ages varies between six months and two years, weight varied between 30gr and 78gr. The purpose of this study was to evaluate the length-dependency of fatigue and recovery, this

was done by comparing active and passive force in different situations, unfortunately it was not possible to look further. In the sense of chemical and metabolic events, for example the activity of Ca^{2+} or SR. A detailed explanation about all findings could not be found. Focus was on peripheral fatigue, because of in vitro-setting, in that way central fatigue was ignored. Although peripheral fatigue took the main part of total fatigue, in every case minimally 30% is due to central fatigue (Bigland-Ritchie et al., 1978). So, to obtain a total image, tests need to be retaken in vivo. The fact that the assessors had no experience, could be a bias and influence the process and results of this experiment.

Based on the findings of the present study, some recommendations could be made for future research. One of them, and maybe most important, is the standardization of the experimental animals. Also the amount of samples should be increased. This in function of power and to create representative results. Further a more extensive research, in terms of more parameters that will be tested, aside of active and passive force, like maximal and minimal force development and relaxation time, respectively. In order to evaluate fibre type difference, a similar protocol has to be used, so results could be compared.

6. Conclusion

Newly developed setup worked and delivered comparable results to earlier research. Used stimulation frequencies did not stimulate both SOL and EDL maximally. Due to a problem with the analysis program, no force-length relationship could be drawn. The main purpose of this study was to evaluate the influence of muscle length on peripheral muscle fatigue. Small effect had been found and only in case of SOL muscle. Future research has to focus on standardization and amount of the available samples.

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8. Appendix

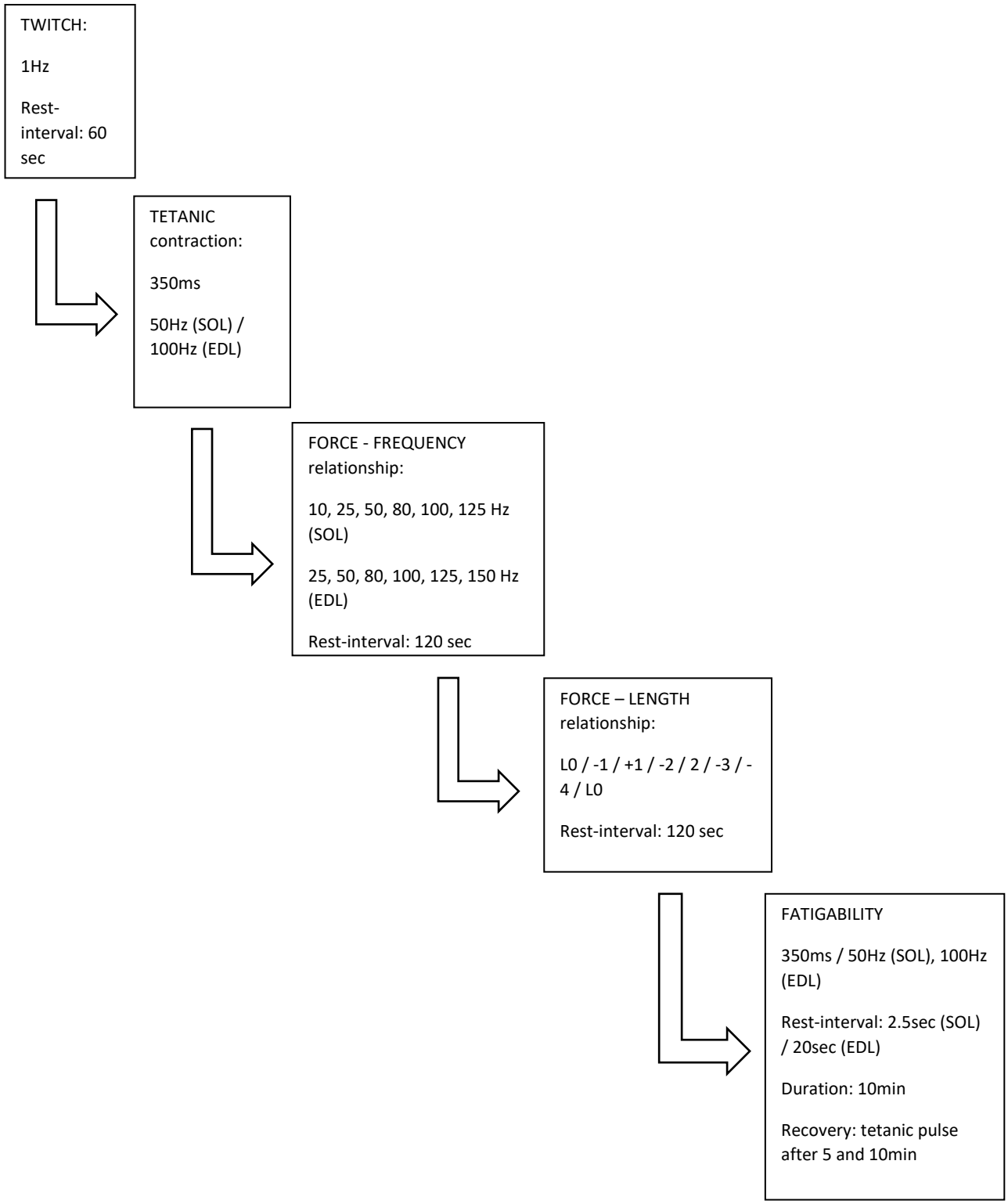

Fig. A: Schematic presentation of the study outline

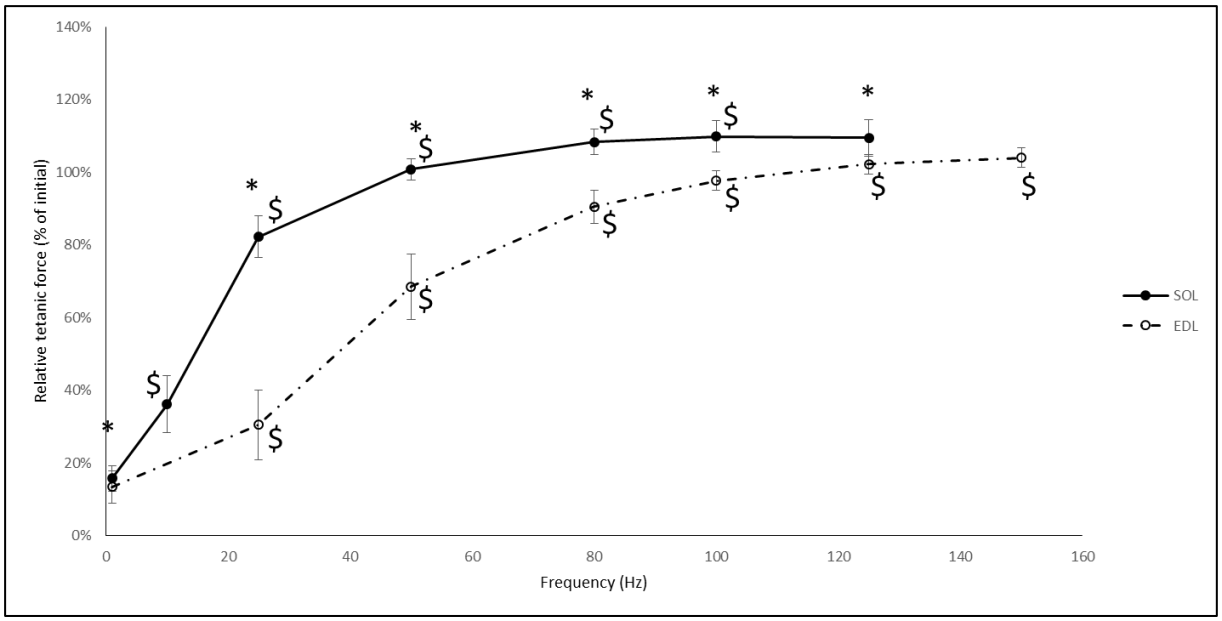

*Fig. B: Force -Frequency relationship (relative to tetanic contraction force). Leftward shift of SOL (soleus) compared to EDL (extensor digitorum longus). *Significant difference between SOL and EDL at certain frequency $ Significant difference relative to previous frequency. p<0.05.*

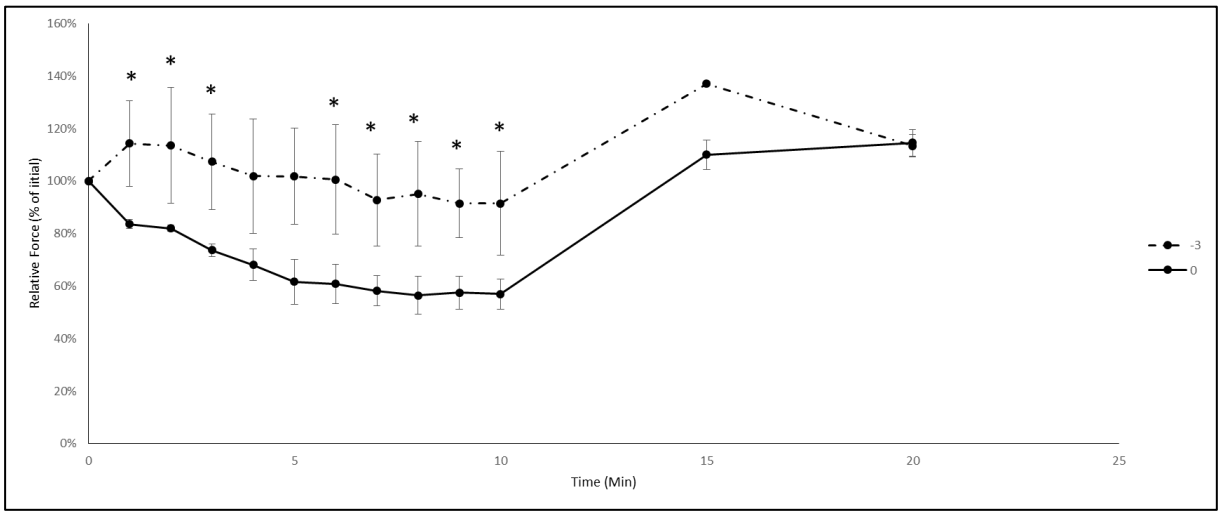

*Fig. C: Difference in fatigability and recovery between L_0 (solid) and $L_{0\text{-}3}$ (dotted) for SOL. Tetanic force (relative to initial force) during 10min of repeated fatiguing stimulation and 10min of passive recovery. *Significant difference to L_0, p<0.05.*

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