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UHASSELT

Faculteit Revalidatiewetenschappen

master in de revalidatiewetenschappen en de kinesitherapie

Masterthesis

Does muscle length effect fatigue in soleus muscle of mice?

Lennart Ghijsens
Lennert Mooren

Scriptie ingediend tot het behalen van de graad van master in de revalidatiewetenschappen en de kinesitherapie,

afstudeerrichting revalidatiewetenschappen en kinesitherapie bij musculoskeletale aandoeningen

PROMOTOR :

dr. Pieter VAN NOTEN

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Acknowledgement

We would like to thank some people for the help and support they gave throughout the past year in order to realize this master thesis.

First of all, we would like to thank our promotor, Doctor-assistant Pieter van Noten, for his time, support, instructions and answers during the past year. Without his help, we would not have been able to succeed in our mission to write this master thesis in our last year!

We also want to thank the University of Hasselt for the possibilities it gave us during our path to accomplishment of this five-year education period. Our special thanks go to the faculty of rehabilitation sciences and physiotherapy for all the knowledge of physiotherapy they have shared with us, aiming to be starting point of a great career.

And last, but not least, we thank our friends and family for the support they gave us. They were there to help us whenever and however they could and played an important role as supporting pillars in good and though times.

Beursstraat 23a 3832 Ulbeek, 03/06/2017   **L.M.**

Okeleistraat 3 3800 Sint-Truiden, 03/06/2017   **L.G.**

Research context

The second part of this master thesis, which is a duo thesis, is an experimental research ('in vitro' study) that fits musculoskeletal rehabilitation. It concerns the effect of fatigue on rate on force production in skeletal mammalian muscles (soleus). The paper is written according to the central format of the 'Rehabilitation Sciences and Physiotherapy' course during the second master year at the University of Hasselt.

It is known that fatigue alters muscle performance. When a tetanic contraction is elicited by an electrical stimulus, force builds up to a maximal peak. As long as fatigue does not occur, the muscle will be able to produce this PF. If the stimulation of the muscle is stopped, the force production will rapidly decrease to zero. This build-up of force is presented in a XY-diagram (i.e. force-time curve) by a rising line and declines after the maximal force is reached. The phase in which force builds up to its peak is characterized by the speed (i.e. the slope of the curve/rising line) at which this PF is achieved. The speed by which a muscle contracts to reach PF, often called rate of force development (RFD) in the literature, is expressed in the amount of force per time interval that can be produced during such a tetanic muscle contraction. Investigations demonstrated that the RFD is slowed in fatigue, but currently it is unclear to what extent fatigue influences and/or changes the RFD in a muscle and if the slowing of the RFD precedes the actual decline in PF during fatigue. This aspect is important for a physiotherapist because slower force production can lead to unbalanced muscle coordination resulting in joint and muscle injuries. Consequently, as muscle fatigue is an important clinical factor, this study aims to evaluate the effect of fatigue on RFD, PF and rise time (RT).

This master thesis is an independent research project, of which the experiments were performed at REVAL (Study Center for Rehabilitation Research) in Hasselt/Diepenbeek.

The research protocol, already described during the first part of the master thesis, is focused on investigating the effect of fatigue on RFD in dissected mammalian soleus muscles. This protocol (i.e. material and method) was developed by the students, based on Everaert, Stegen, Vanheel, Taes, and Derave (2013) and Chen, Hothi, Xu, and Huang (2007).

After agreement of the intended parameters to measure and observe during data-extraction, the students worked together as a team to conduct the experimental research. Hereafter, the obtained results were bundled all together. Finally, a critical discussion was written based on

these results. All the experiments and proceedings were performed under supervision of doctor-assistant Pieter Van Noten.

Each student has done the same number of hours and amount of work to bring the master thesis to a good end.

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1. Abstract

Background:

Muscle fatigue is a physiological process that is not only characterized by a recoverable force loss, but also by a slowed rate of force development (RFD). Because of its physiological character, parameters that represent these processes will probably be more affected. Muscle length influences fatigue and thus possibly also RFD.

Material and method:

After dissection, 40 soleus muscles were randomly assigned to a specific length (L_{0-2}mm; L_{0-1}mm, …) at which they were fatigued (350ms tetanic contractions at 50Hz every 2,5s for 10 minutes). Before the fatiguing protocol, muscles were tested for the complete force-length relation and were evaluated at their assigned length for twitch and tetanic contraction, and force-frequency relation. Then, fatigue and recovery (five and ten minutes after fatigue) were assessed. Maximal active force (i.e. peak force ~ PF) and RFD were analyzed for all contractions.

Results:

Results of the force-length protocol showed that both PF and RFD were optimal at L_0. During the force-frequency protocol, PF and RFD increased up until 100Hz for all lengths. PF and RFD both declined during fatigue for all lengths, but mostly in the beginning. The rate of decline in PF and RFD was different between all muscle lengths and showed to be higher at the ascending limb and plateau of the force length relation compared to the descending limb (via graphical analysis). After five and ten minutes of recuperation, both PF and RFD recovered for all lengths, but recovery to initial values was not reached.

Conclusion:

Muscle length influences force production, rate of fatigue and RFD. Fatigue is greater at optimal and short muscle lengths than it is at longer muscle lengths. Also, RFD probably declines faster during fatigue than PF does. These results can have clinical importance, but further research should be done to confirm these results and to understand the underlying mechanisms.

2. Introduction

Human locomotion describes the act of moving, which requires muscles to generate a rotational force around a joint to complete a task (Fitts, McDonald, & Schluter, 1991). When movements are performed for a long time or are extremely exhausting it can lead to muscle fatigue. Muscle fatigue is caused by a number of physiological events that result in an altered muscle contraction and is a process in which force is gradually lost (i.e. inability to maintain maximal force production) (Wang, Qin, Wang, Sun, & Liu, 2017). Consequently, execution of tasks and movements will no longer be optimal.

There are three phases in muscle fatigue: during the first two phases, fatigue causes a reduction in myofibrillar force-producing capacity (i.e. cross-bridge cycle) to 80-90% of PF, but no reduction the number of cross-bridges. There is one main component responsible for this event, namely an increase in P_i in combination with a decrease of P_{Cr}. In the last phase, mainly a reduced Ca^{2+} release from the sarcoplasmic reticulum causes the number of activated cross-bridges to decline and causes a decrease to 30-40% of PF. These mechanisms of fatigue lead to a serious drop in force production (Allen, 2009; Lännergren, Westerblad, & Allen, 2006). By understanding the molecular mechanisms of the cross-bridge cycle (Fig. 1) in an unfatigued state, it can be understood how functional changes result from direct effects on the myofilaments during fatigue.
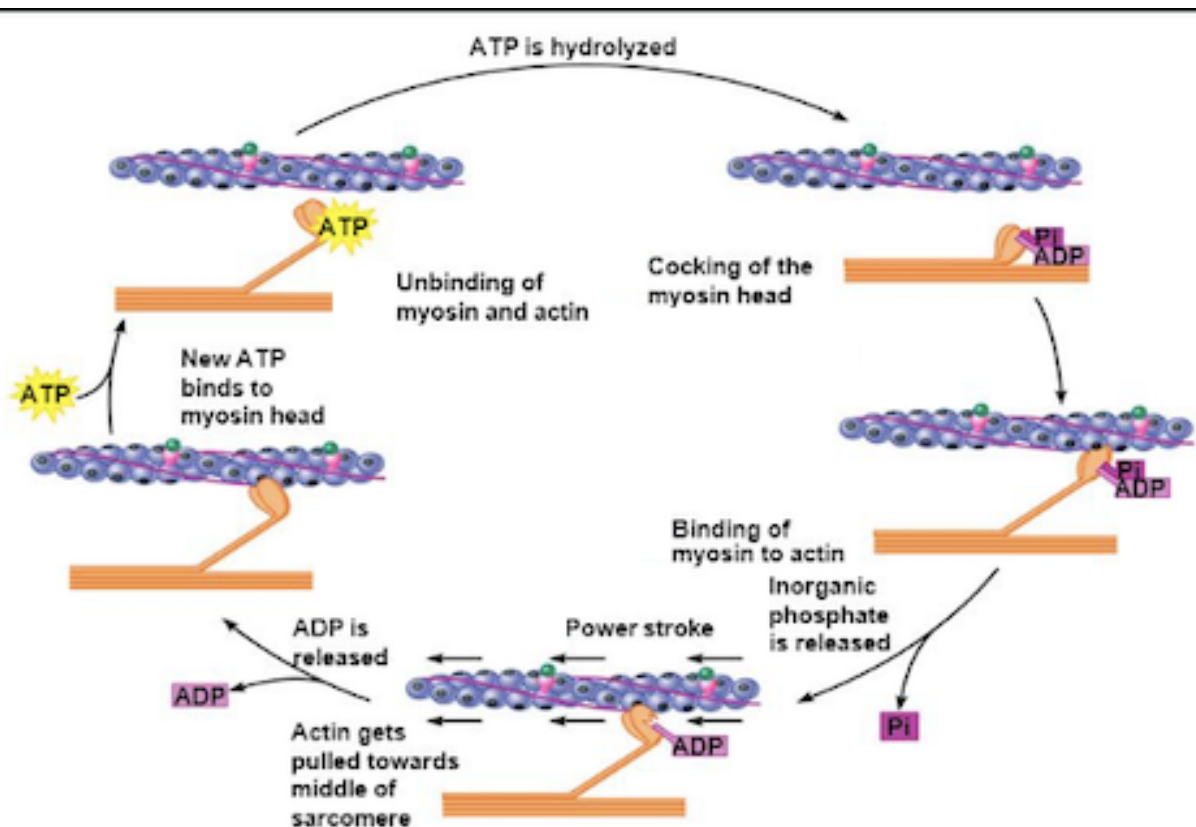
Figure 1: The cross-bridge cycle. Adapted from Principles of Human Physiology (p. 328), by C. L. Stanfield, 2013, San Francisco, CA: Pearson Education. Copyright 2013 by Pearson Education, Inc. Schematic Model presenting the physiological processes during a cross-bridge cycle to produce force: Calcium induces movement of tropomyosin-complex, thereby uncovering the active binding sites of actin to myosin (not shown). Adenosine phosphate (ATP) binds to the myosin head in order to dissociate the actin-myosin complex. Next, the myosin head is activated by hydrolyzing the attached ATP into adenosine diphosphate (ADP) and phosphate (P_i). This causes myosin to go into the cocked position. Hereafter, the activated myosin binds on actin to form a weakly bound low-force state and subsequently P_i is released to make the actin-myosin cross-bridge strongly-bound and the filaments slide past each other during the following power stroke due to release of ADP (Lundy-Ekman, 2013; Greising, Gransee, Mantilla, & Sieck, 2012). The moment a myofilament of the cross-bridge is in a strongly-bound high force state, it determines maximal isometric peak force of that myofilament (Fitts, 2008).

In the presence of fatigue, the decrease in isometric PF can be explained by a decrease of the force per cross-bridge and/or the number of cross-bridges in these high-force states (Nocella et al., 2011). As already mentioned, the first signs of fatigue result from physiological changes that affect the cross-bridge cycle, by which the PF decline is only a small result. In the last phase, loss in PF is most prominent because of the decrease in number of cross-bridges. Indeed, Andersen and Aagaard (2006) indicated that isometric PF is a balance between building and breaking down of cross-bridges, whereas RFD is the expression of the coherence

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between all the physiological processes that might indicate fatigue in the initial stage. More specifically, the transition speed from the weakly bound low-force state to the strongly bound high-force state by the release of P_i is thought to determine RFD (Fitts, 2008). The slowing down of RFD could predominantly be caused by an increase in P_i and a decrease in P_{Cr} during the first phase of fatigue. When the excess amount of P_i cannot be removed by P_{Cr}, the increase of P_i is thought to reduce RFD by slowing down the transition from a weakly bound to a strongly bound myofilament or even accelerate the reverse rate constant (i.e. from strong bound to weakly bound) (Allen, 2009; Lännergren, Westerblad, & Allen, 2006; Allen, Lännergren, & Westerblad, 1995; Fitts, 2008). Consequently, RFD will decrease/slow down because there is a slower (i.e. longer) time-course of contraction during fatigue. While RFD takes in account the force-increase within a certain time interval, rise time (RT) will solely consider the time interval.

Following the aforementioned assumption that fatigue is the result of physiological events during cross-bridge interactions, it can be concluded that fatigue is related to the amount of actin-myosin overlap. Cross-bridge formation can only occur at the sites of actin-myosin overlap and therefore, maximal isometric PF is positively related to the amount of myofilament overlap (Rassier, MacIntosh, & Herzog, 1999). Thus, greater fatigue should be observed at optimal muscle length because overlap is optimal and the amount of cycling cross-bridges and metabolic rate (including P_i) is maximal. When a muscle is lengthened or shortened, the sarcomere length changes proportionally resulting in an alteration (i.e. increase or decrease) in overlap of the actin-myosin complex. This results in a decrease in PF production (Fig. 2). To support this theory, Fitch and McComas (1985) tested the effect of muscle length on susceptibility to fatigue in the dorsiflexor muscles of human, using a fatiguing protocol that consisted of a 90-second during tetanic stimulation at 20Hz. Two lengths were used, namely 15° plantar flexion (i.e. optimal length ≈ maximal lengthened due to articular restrictions) and 25° dorsiflexion (i.e. fully shortened). They found a significant reduction of twitch and tetanic torque at optimal (≈ long) length following fatigue, but not at the shortened length. Lee et al. (2007) also tested this theory in a similar way by stimulating the quadriceps in vivo with 40Hz during 180 seconds at short (i.e. 15° knee flexion) and optimal/long (90° knee flexion) muscle lengths. They concluded that at optimal/longer muscle length, fatigue

was greater. To our knowledge, no studies investigated or revealed the length dependence of RFD during fatigue.
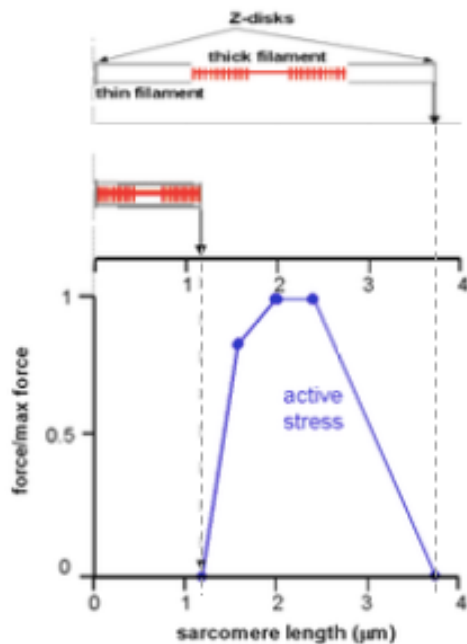
Figure 2: Force-length relationship of skeletal muscle sarcomere (Langton, 1999).
The upper part represents the amount of actin-myosin overlap in a sarcomere. The lower part is a representation of maximal force correlated to the sarcomere length: both short and long sarcomere lengths cause a decrease in maximal force production.

As a conclusion, it is important to investigate the effect of physiological muscle fatigue on force characteristics, PF as RFD and RT, at different muscle lengths. With this in mind, the length-tension relation can be easily measured and investigated in vitro under conditions where length of the muscle can be precisely altered over a wide range (Langton, 1999). In this study, experiments will be performed on the soleus muscles of mice, which consists predominantly of type I muscle fibers (96.1%±2.9) (Soukup, Zacharová, & Smerdu, 2002). The goal of this study is to investigate the effect of fatigue on force production at different soleus muscle lengths as the question remains what influence these changes in muscle length have on PF, RFD and RT. By assessing PF and RFD during a fatiguing protocol, it will be able to determine which factor is most valuable to assess fatigue on muscle level.

3. Material and Method

3.1 Method of measurement-analysis

3.1.1 Ethical approval

The study was approved by the UHasselt 'Ethical Commission Animal Tests'.

3.1.2 Animals

All animals used were male mice who had food and water ad libitum. They were held in a room with a day-night cycle of 12 hours at a temperature of 19-20°C. The mice were at the age of 13 months and their mean weight was 43gr.

An intraperitoneal injection with pentobarbital was used to anaesthetize the mice. Muscles were dissected, whereafter animals were euthanized by cervical dislocation.

3.1.3 Muscle and measurement-analysis preparation

Soleus (SOL) muscle was mechanically dissected, according to Park, et al. (2012). Platinum plates were flanking the muscles to evoke field stimulation and their tendons were attached between a force transducer and a micro-positioner in 13ml Krebs-Henseleit solution (117mM NaCl, 25mM $NaHCO_3$, 5mM KCl, 1mM $MgSO_4$, 1mM KH_2PO_4, 2.5mM $CaCl_2$, 0.5mM glucose). This solution was continuously gassed with 95% O_2 and 5% CO_2, and held at a constant temperature of 25°C (Everaert et al., 2013).

Field stimulation took place in the following manner: a HSE stand-alone stimulator generated square-wave pulses. An isolation unit filtered these pulses to reach the platinum cord that surrounded the isolated muscle-tendon. Muscle contractions responding to the stimulation were recorded by the force transducer and sent back to the computer (Fig 3). These measurement values were saved for later analyses (Park et al., 2012).

Optimal muscle length (L_0) was determined using a micro-positioner and tetanic stimulations with 350ms train-duration, 50Hz frequency and a 2-minute rest interval. In the beginning, the muscle was lengthened until the passive force contribution initiated (5mN). After each stimulation, muscles were lengthened by 0.5mm to identify the optimal muscle length (i.e. the point where active force production was maximal) (Park et al., 2012; Everaert et al., 2013).
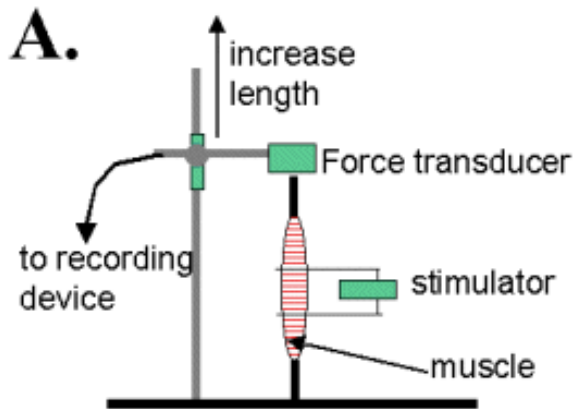
Figure 3: Experimental set-up to determine length-tension relation of skeletal muscle (Langton, 1999).

3.1.4 Protocol

A total of 40 soleus muscles were dissected and randomly assigned into five groups, related to the particular length at which the fatiguing protocol was performed: at optimal length (L_0), at 1mm and 2mm short of L_0 (L_{0-1} and L_{0-2}), and at 1mm and 2mm longer than L_0 (L_{0+1} and L_{0+2}). Each muscle passed the whole stimulation protocol after optimal muscle length was determined. Optimal length was the length at which maximal force can be produced. The stimulation protocol consisted of five parts, carried out in the following order: evaluating muscle length, twitch and tetanic response, force-frequency relation, a fatiguing stimulation and recuperation.

Muscles were stimulated at a frequency of 50Hz with a pulse-duration of 350ms to produce a tetanic contraction for the whole protocol, except for the force-frequency relation (see 3.1.4.3)

3.1.4.1 Testing different lengths

After the determination of L_0, the muscle was adjusted to six muscle lengths (of which five different from L_0) in order to have a quick test of its influence on the three intended parameters. The muscle was positioned and stimulated at a certain length. Hereafter, a 2-minute rest interval was given, and the muscle was positioned at the next length. This process was executed in the following order: L_{0-2}, L_{0+1}, L_{0-3}, L_{0+2}, L_{0-1} and L_0. For each length, one tetanic contraction was given.

3.1.4.2 Twitch and tetanic contraction

The muscle was adjusted to a definite length according to the group it belonged to. By stimulating the muscle with one twitch contraction and one tetanic contraction, reference values were measured to obtain a baseline for PF, RFD and RT.

3.1.4.3 Force-frequency relationship

Obtaining the force-frequency relationship was important to identify the stimulation frequency at which maximal tetanic force could be generated, i.e. a plateau in force. As a result, the proportion of type I muscle fibers in the soleus muscles could be determined. This part of the protocol also showed how frequency influenced PF, RFD and RT at different lengths. Therefore, the soleus muscles were stimulated with the following frequencies: 10Hz, 25Hz, 75Hz, 100Hz and 125Hz. Type I muscle fibers are optimally stimulated at 50Hz. Thus, when a force plateau is seen, it could indicate the dominance of fiber type in the investigated soleus muscles.

### 3.1.4.4 Fatiguing protocol

This part of the protocol was carried out to fatigue the muscle. Fatigue was determined by the percentage decrease in tetanic force in comparison to the initial force. The main goal of this protocol was to analyze the effect of fatigue on PF, RFD and RT at different lengths, so it could be linked to the physiological reactions that occur during muscle fatigue. The total stimulation duration to reach fatigue was 10 minutes of repeated tetanic contractions with each contraction consisting a train duration of 350ms, 50Hz frequency every 2.5s for soleus muscle.

3.1.4.5 Recuperation

Five and 10 minutes after fatiguing stimulation, a tetanic stimulation was given to measure the recuperation of the muscle.

3.2 Specific parameter analysis

The parameters that were used to extract information of a muscle contraction before, during and after fatigue, were rate of force development (RFD), rise time (RT) and peak force (PF). The first two measurements were carried out in a 20-80% range of the rising phase of the contraction (Fig 4).
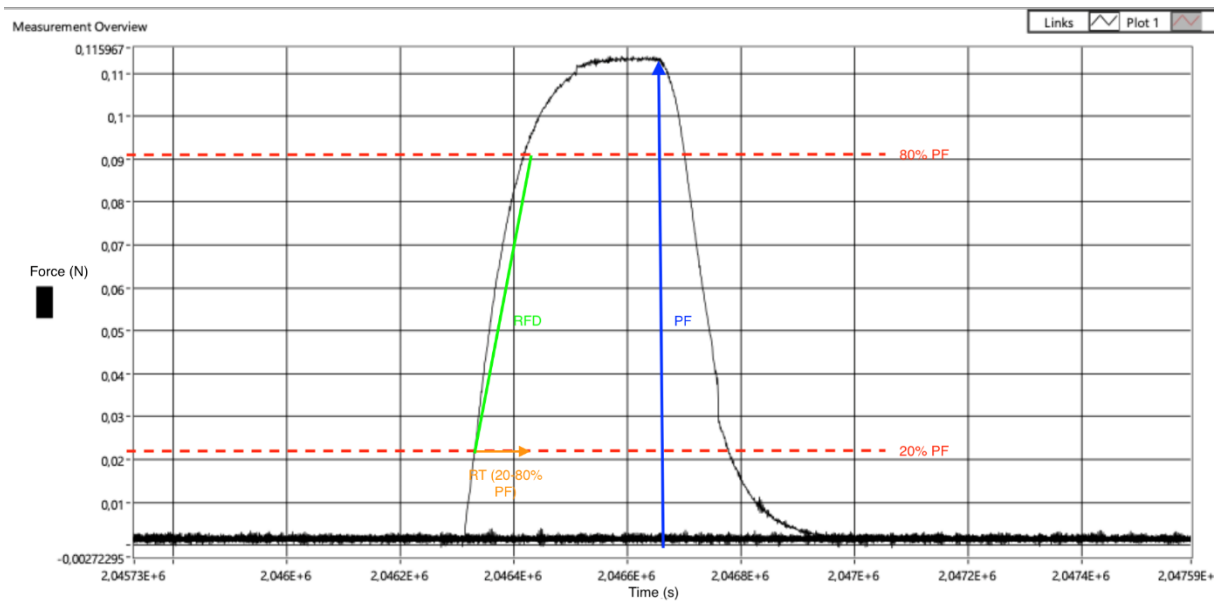
Figure 4: Graph of a force-time curve. An example of a force (Y-axis in N) time (X-axis; seconds) curve of a tetanic contraction of a soleus muscle is presented in black. A plateau in force production can clearly be distinguished. Maximal active force, also called peak force, is indicated in blue (Peak Force (PF) = Maximal force – passive force). Two parameters were used to describe force development from 20-80% of PF: Rate of force development (RFD = ΔForce/ΔTime) in green and Rise Time (RT) in orange.

3.3. Statistics

Data are presented as mean ± SD and as mean% ± SD. Normality was investigated for all the datasets by the Shapiro-Wilk Test. The test showed that data was not distributed normally (p<0,05). Therefore, non-parametric statistics were used. Data was submitted to 'JMP Pro 14' and matched pairs statistics were used to determine statistically significant differences between groups (muscle lengths) and outcome measures (PF, RFD 20-80% and RT 20-80%). Level of significance was set at α<0,05.

4. Results
4.1 Force-length

Stimulation of all muscle lengths led to the following result: L_0 (rest length) was the length at which maximal active force production could be reached. PF at L_0 was maximal and significantly higher ($p < 0.05$) in comparison to other lengths (Fig 5). RFD was also maximal and significantly higher ($p < 0.05$) when the muscle was stimulated at length L_0. This showed that L_0 is the optimal length to elicit maximal force production.
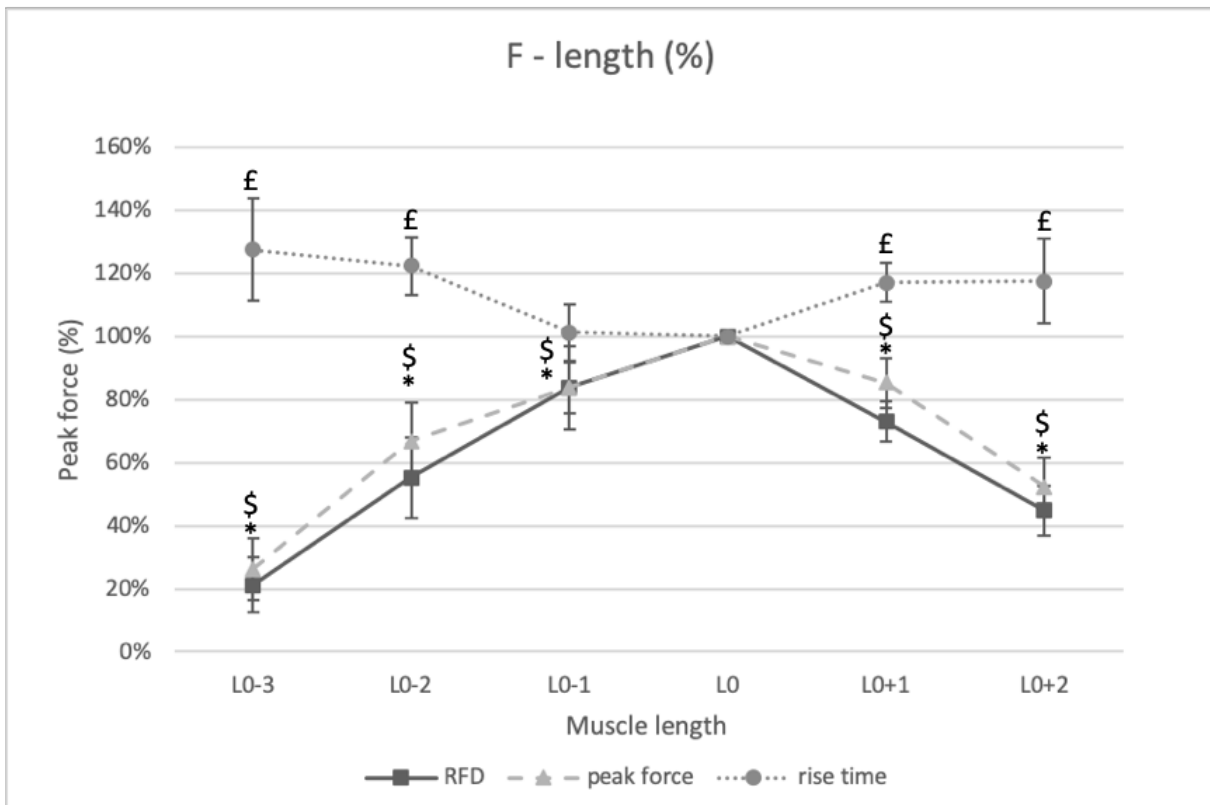
**Figure 5: Force – length protocol (relative values)**

Mean % ± SD for PF, RFD and RT during F-length protocol. $: $p < 0.05$ for PF compared to L_0; *: $p < 0.05$ for RFD compared to L_0; £: $p < 0.05$ for RT compared to L_0.
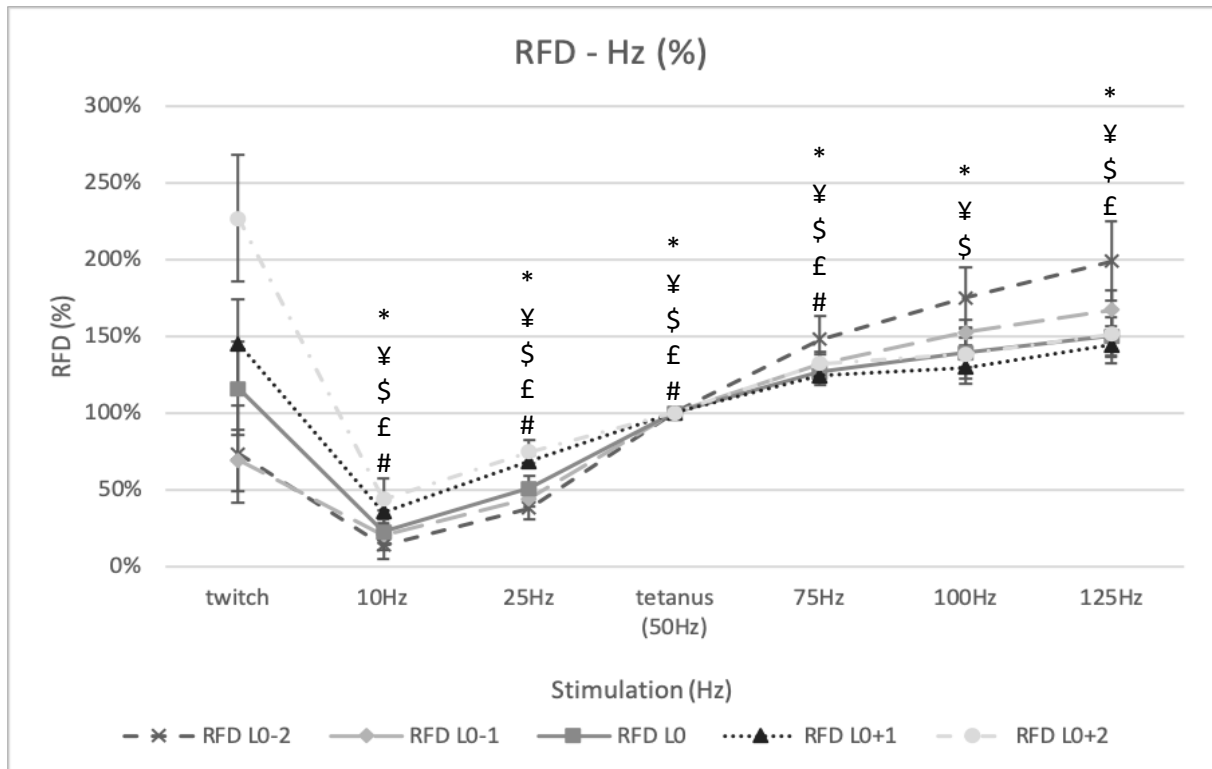
4.2 Force-frequency

Figure 6 shows that PF increased significantly with increasing stimulation frequency up until 100Hz ($p<0.05$). When the stimulation frequency rises further up to 125Hz, PF decreased compared to stimulation at 100Hz for L_0, L_{0+1}, L_{0-1} and L_{0-2} ($p<0.05$). For L_{0+2}, no statistical difference was measured at 125Hz.
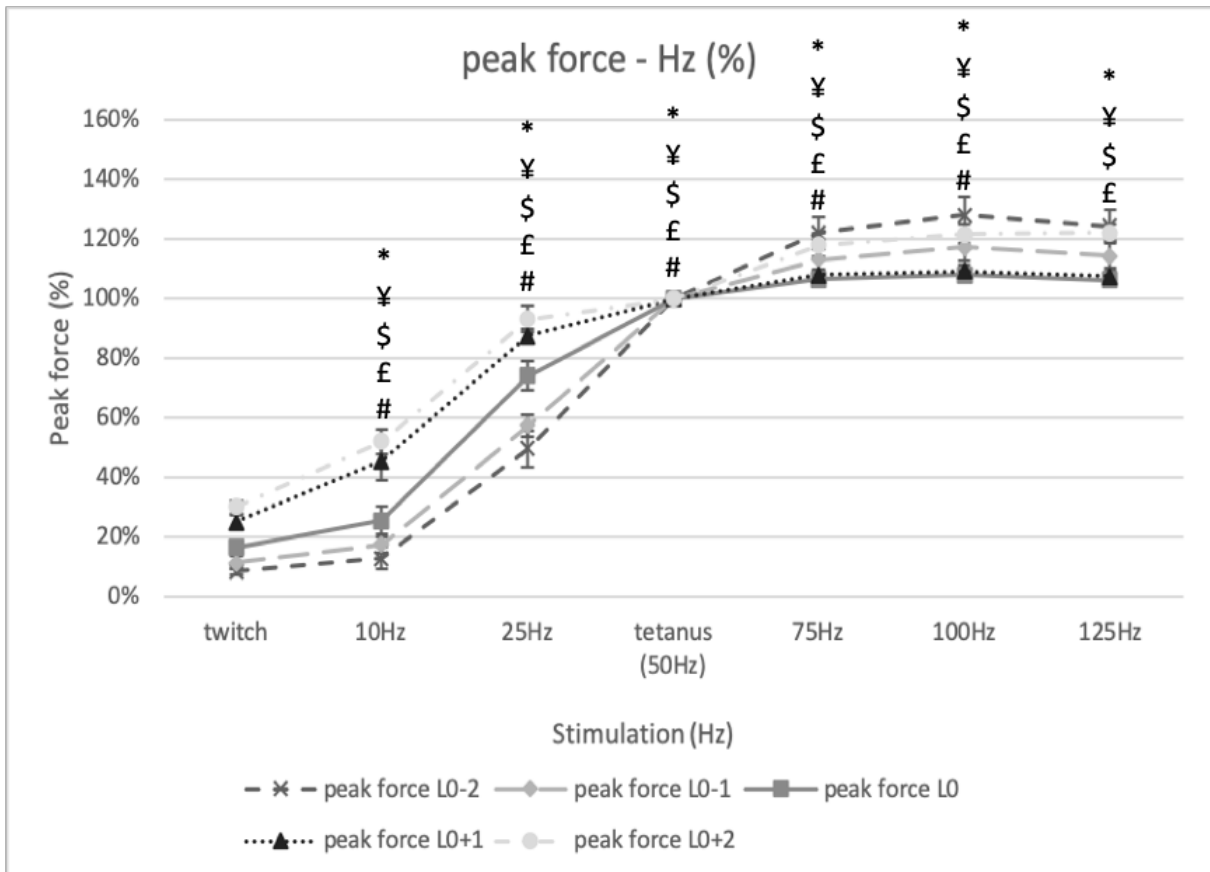
Figure 6: Peak force – frequency protocol for all lengths

Mean % ± SD for PF during F-Hz protocol for all lengths. *: $p<0.05$ for PF compared to lower frequency for L_{0-2}; ¥: $p<0.05$ for PF compared to lower frequency for L_{0-1}; $: $p<0.05$ for PF compared to lower frequency for L_0; £: $p<0.05$ for PF compared to lower frequency for L_{0+1}; #: $p<0.05$ for PF compared to lower frequency for L_{0+2}.

RFD was significantly higher in twitch contractions, compared to 10Hz stimulation for all lengths ($p<0,05$). RFD kept rising significantly from 10 Hz until 125Hz for L_0, L_{0-1} and L_{0-2} ($p<0.05$). For L_{0+1} and L_{0+2} two exceptions were found: RFD did not increase significantly from 75Hz to 100Hz for L_{0+1} and L_{0+2}, and from 100Hz to 125Hz for L_{0+2} alone (Fig. 7).

Figure 7: Rate of Force Development – frequency protocol for all lengths

Mean % ± SD for RFD during RFD-Hz protocol for all lengths. *: $p<0.05$ RFD compared to lower frequency for L_{0-2}; ¥: $p<0.05$ for RFD compared to lower frequency for L_{0-1}; $: $p<0.05$ for RFD compared to lower frequency for L_0; £: $p<0.05$ for RFD compared to lower frequency for L_{0+1}, #: $p<0.05$ for RFD compared to lower frequency for L_{0+2}.

4.3 Fatigue

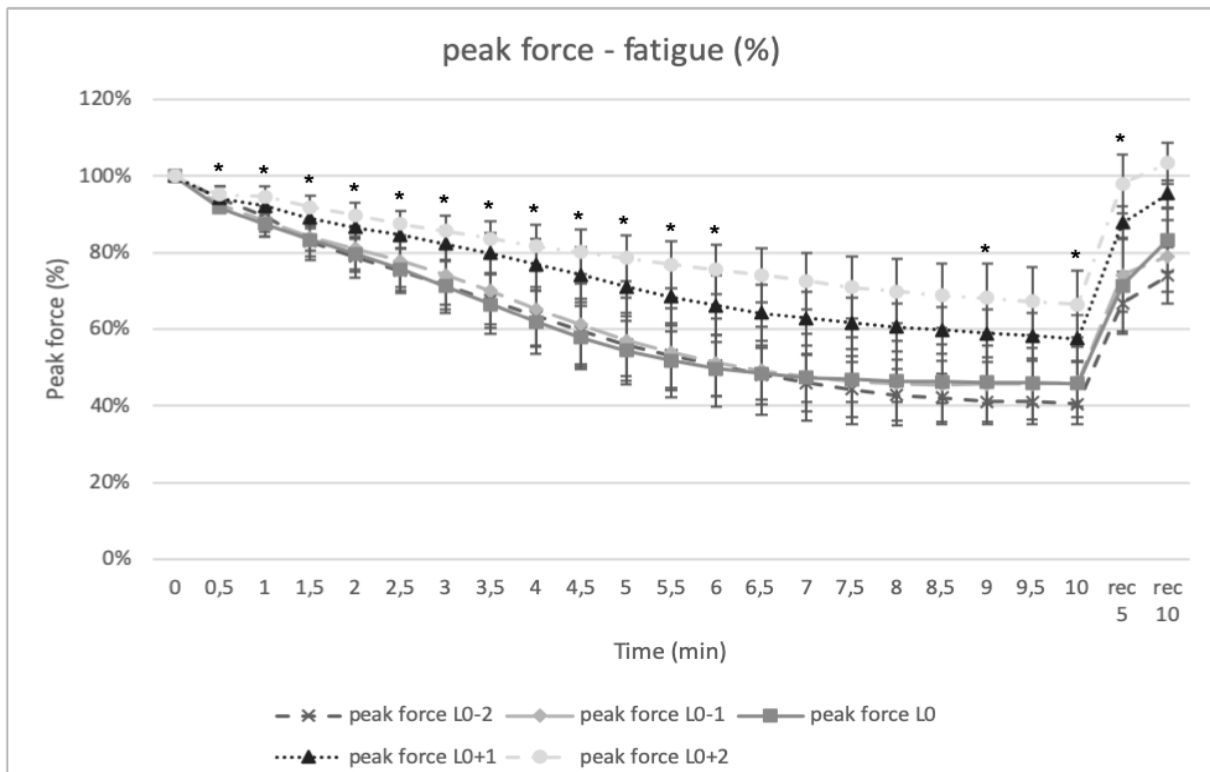**4.3.1 Fatigue: Peak force**

When analyzed for all lengths combined, PF declined throughout the whole fatiguing protocol (p<0.05) (Fig. 8). When analyzed separately, PF decline continued until 7.5 minutes for each length (p<0.05). For L_0, L_{0-1} and L_{0-2} there was no change in PF after 7.5, 8 and 9 minutes respectively. For the long muscle lengths (i.e. L_{0+1} and L_{0+2}), PF declined significantly during the whole fatigue protocol (p<0.05). PF mean responses between different muscle lengths differed during the whole fatiguing protocol (p<0.05) (Fig. 8). Also, the PF decline was significantly different between all lengths until 6 minutes (p<0.05), with a graphical analysis showing more decline at L_0, L_{0-1} and L_{0-2} compared to L_{0+1} and L_{0+2} (Fig. 9). Hereafter, PF decline was the same for different lengths at most measurements (Fig. 9). During recovery for all lengths combined, PF increased after 5 minutes and after 10 minutes (Fig. 8). The increase of PF during recovery was different between lengths at 5 minutes into recovery (p<0.05), but not at 10 minutes recovery (Fig. 9). Recovery to initial values was not reached (p<0.05).
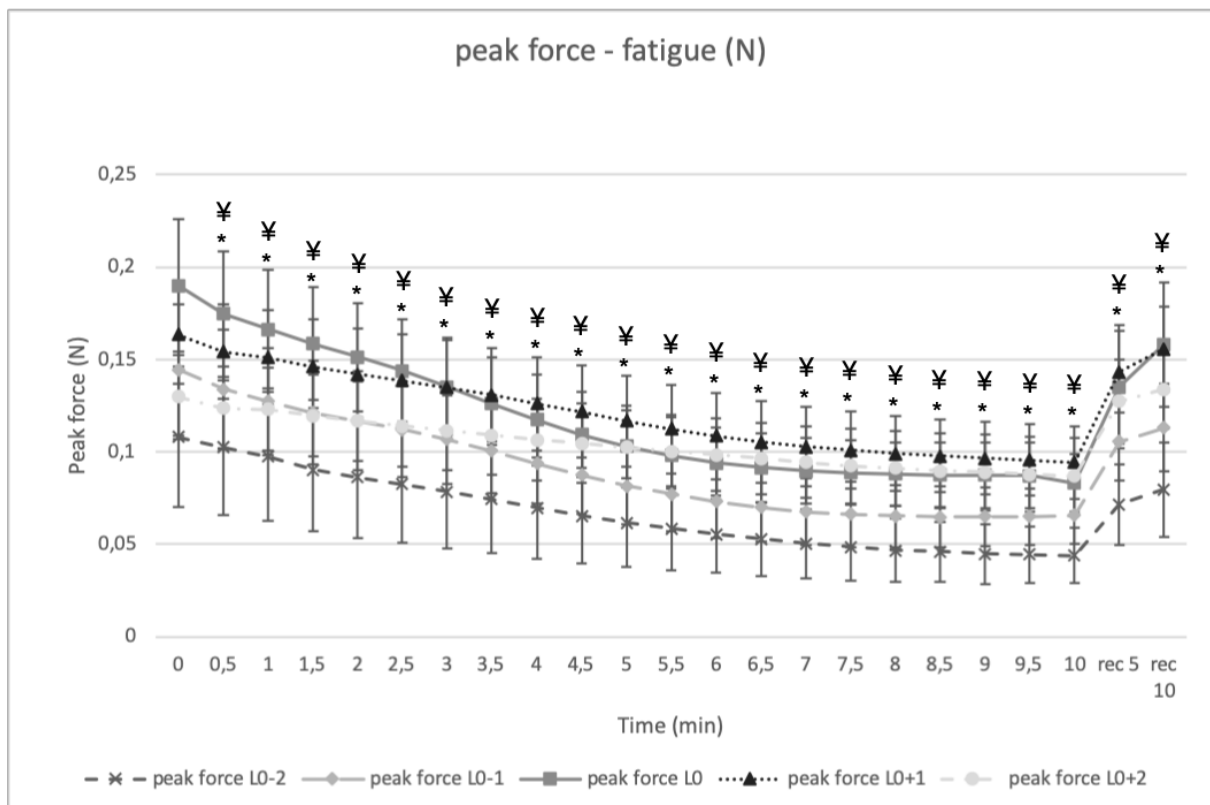
Figure 8: Peak force – fatigue (N) for all lengths

Mean ± SD for PF during fatiguing protocol for all lengths. *: p<0.05 for PF compared to previous measure point for all lengths combined, ¥: p<0.05 for PF between lengths at measure point.

Figure 9: Peak force – fatigue (%) for all lengths

Mean % ± SD for PF during fatiguing protocol for all lengths. *: p<0.05 in change of PF for different lengths between measure points.

4.3.2 Fatigue: Rate of Force Development

The same analyses were made for RFD. RFD declined until 6.5 minutes for all lengths combined (p<0.05) (Fig. 10). After this there was no further decline, except at some measure points (Fig. 10). During recovery, RFD increased after 5 minutes and after 10 minutes (Fig. 10). The increase of RFD during recovery was different between lengths at 5 minutes into recovery (p<0.05), but not at 10 minutes recovery (Fig. 11). Recovery to initial values was not reached (p<0.05).

RFD was also analyzed for each length separately. RFD decreased (p<0.05) every 30 seconds until 6 minutes for L_{0-2} and L_{0-1}, 6.5 minutes for L_0 and L_{0+1}, and 5 minutes for L_{0+2}. From these points, the graph showed a plateau for all the different muscle lengths until the end of the fatiguing protocol. Although there were sometimes still differences in the plateau (p<0.05), RFD remained constant in general.

Mean responses of RFD differed at each measure point during the whole fatiguing protocol between different lengths (p<0.05) (Fig. 10). Until 4 minutes into the protocol, the decline of relative RFD was different between lengths (p<0.05), with a graphical analysis showing more

decline at L_0, L_{0-1} and L_{0-2} versus L_{0+1} and L_{0+2} (Fig. 11). During the rest of the fatiguing protocol, the changes in RFD were not different between lengths anymore.
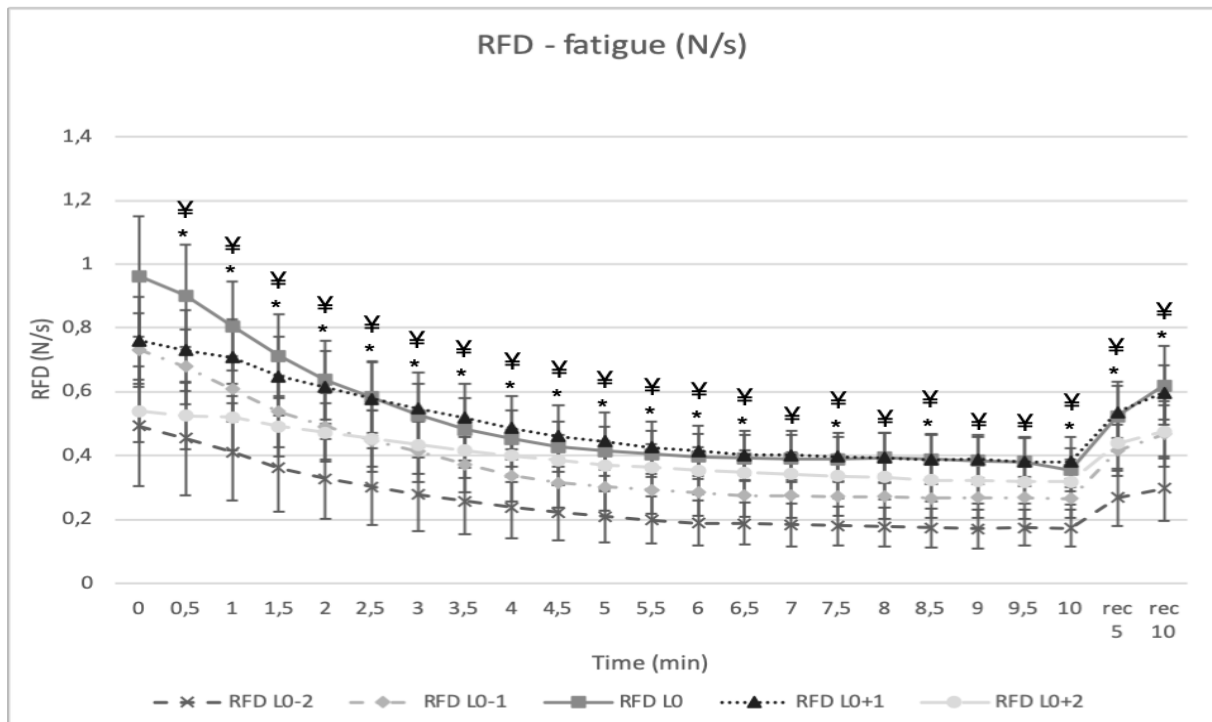
Figure 10: RFD – fatigue (N/s) for all lengths

Mean ± SD for RFD during fatiguing protocol for all lengths. *: p<0.05 for RFD compared to previous measure point for all lengths combined, ¥: p<0.05 for RFD between lengths at measure point.
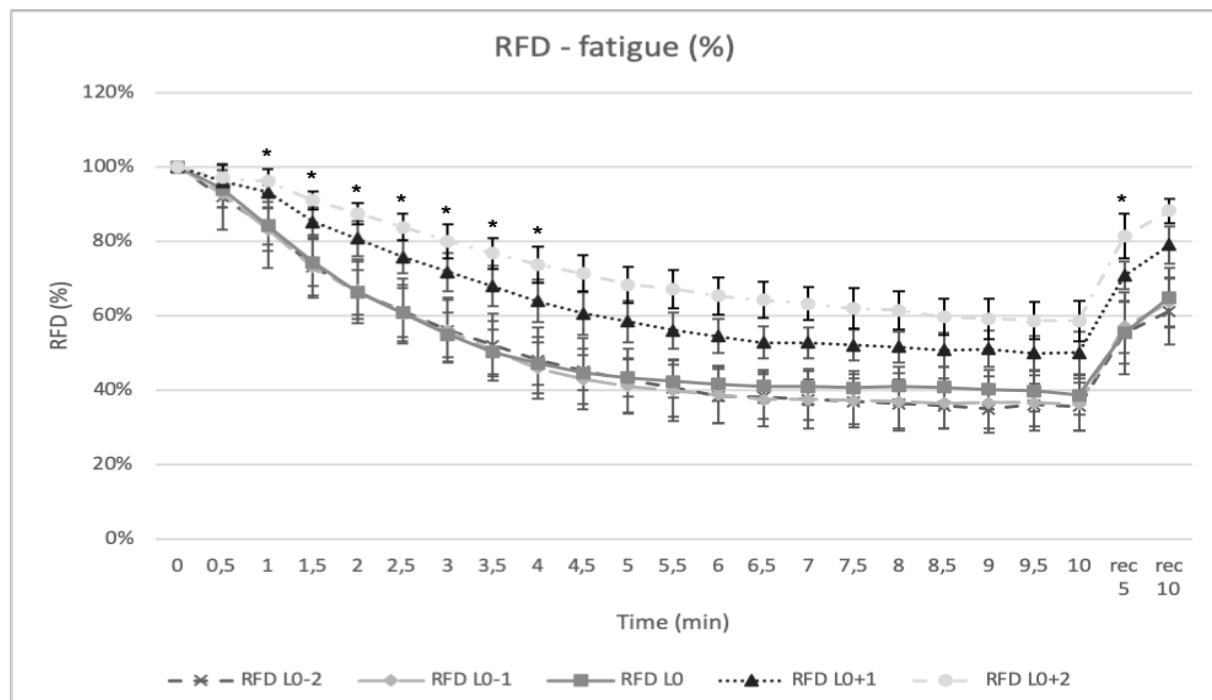
Figure 11: RFD – fatigue (%) for all lengths

Mean % ± SD for RFD during fatiguing protocol for all lengths. *: p<0.05 in change of RFD for different lengths between measure points.

4.3.3 Fatigue: Rise time

RT increased from 1 minute until 4 minutes for all lengths combined (p<0.05). After this, RT decreased at 5.5, 6.5, 7 and 7.5 minutes for all lengths combined (p<0.05). From there, RT remains constant until recovery. At 5 minutes recovery, RT increased compared to 10 minutes fatigue (p<0.05). At 10 minutes recovery RT decreased compared to 5 minutes recovery (Fig. 12) (p<0.05).
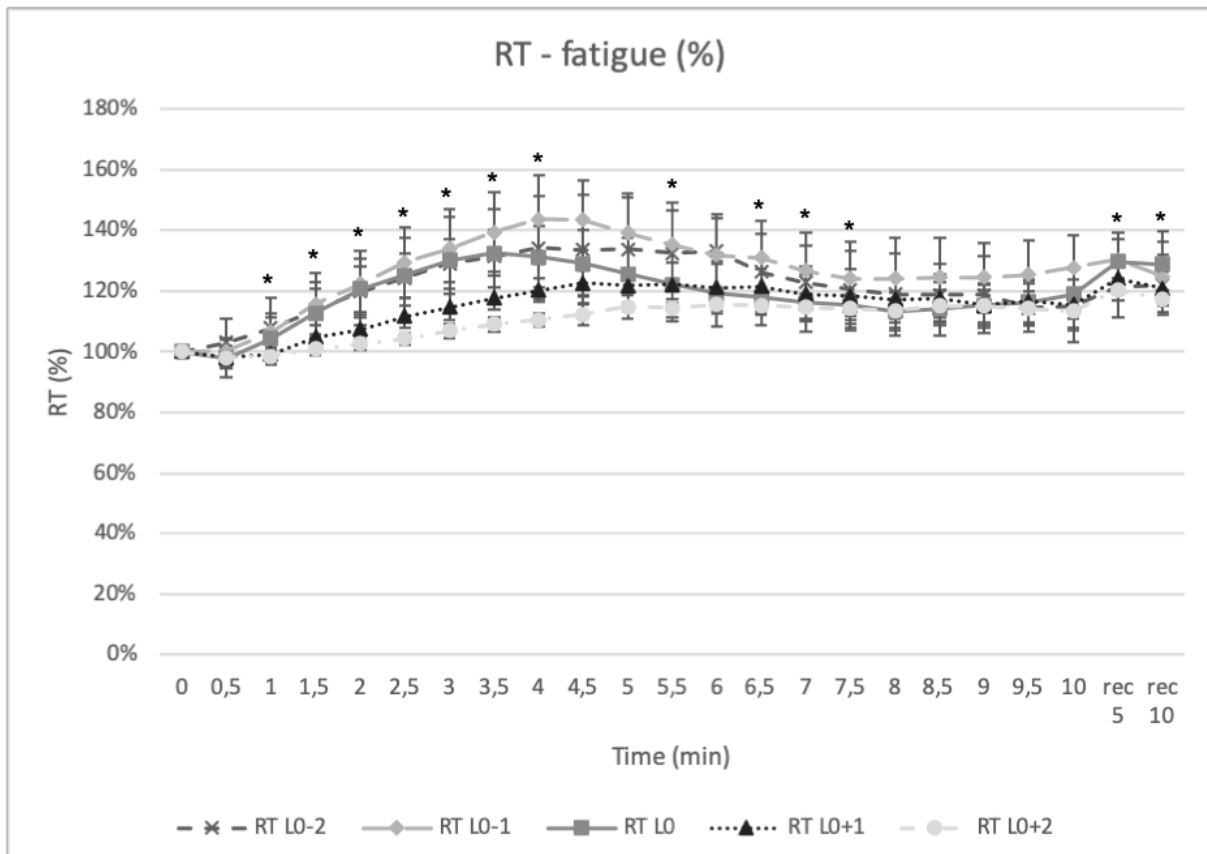
Figure 12: RT – fatigue for all lengths

Mean % ± SD for RT during fatiguing protocol for all lengths. *: p<0.05 RT compared to previous measure point for all lengths combined.

5. Discussion

5.1 Force-length

The results of this study showed that PF was optimal (i.e. maximal) at L_0. At all other lengths PF was less optimal/maximal. This is due to the fact that there is relationship between muscle length and the amount of force production. This amount of force production depends on the number of cross-bridges that are formed in each sarcomere of the muscle (Gordon, Huxley, & Julian, 1966). RFD was also highest at L_0. The more (both shorter and longer) muscle length differed from the optimal length, the more RFD declined during stimulation. These changes cannot be explained by the number of cross-bridges formed at different lengths, because RFD is a result of physiological changes that affect the cross-bridge cycling (Andersen & Aagaard, 2006; Fitts, 2008). A possible explanation for the decline of RFD at lower lengths is that there is little passive tension on the muscle-tendon complex. The muscle-tendon complex has a certain viscoelasticity. This elasticity must be included during a muscle contraction, so it will take the muscle longer to reach PF (Maffiuletti et al., 2016). The reason for the decline in RFD at longer muscle lengths may be due to the greater passive resistance the muscle must overcome with a lower PF (Wilkie, 1949). If the force-length curve of this study is compared to the force-length curve below (Fig. 13), sarcomere length for the different muscle lengths can be estimated. For L_{0-1} PF declined to $\pm$ 80% of the optimal length. According to figure 13, sarcomere length will be around 1.6µm. For L_{0-3} PF declined to $\pm$ 20% which compares to a sarcomere length of $\pm$1.2µm. For the longer lengths the sarcomere length will be between 2.6µm an 3µm according to the figure below. Although, this reasoning should be approached with caution, because (to our knowledge) the effect of change in muscle length on sarcomere length has not been clearly investigated.
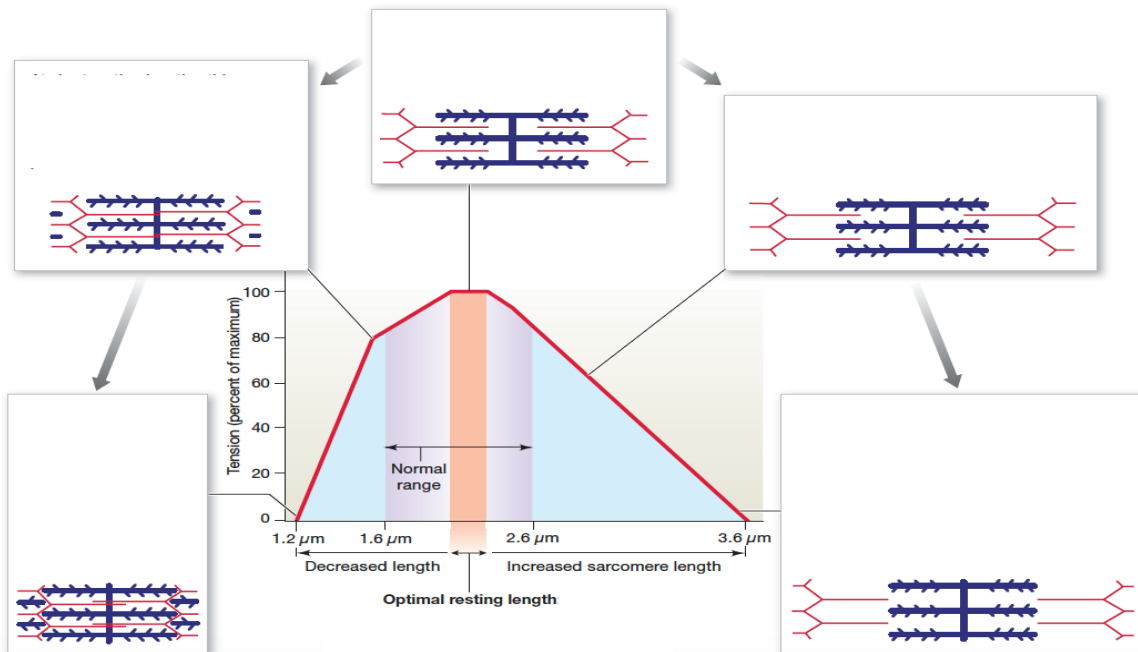
Figure 13. The effect of sarcomere length on active tension. Adapted from *Fundamentals of Anatomy and Physiology* **(p. 298), by F. H. Martini, 2012, San Francisco, CA: Pearson Education, Inc. Copyright by F. H. Martini, Inc., J. L. Nath and E. F. Bartholomew, Inc.**

Force – length relationship at sarcomere level: percentage of maximal force for different sarcomere lengths. Overlap of actin and myosin is shown for the different sarcomere lengths (Martini, 2011).

5.2 Force-frequency relation

Results showed that RFD was much higher during twitch contraction than it is at 10Hz. This could be attributed to the fact that pulse duration of the twitch contraction was much shorter than the contractions at 10Hz.

From 10Hz up until 50Hz the increased in PF is steep. A stimulation frequency above 50Hz showed a graphic plateau in force production, although PF continued to increase significantly for all lengths until 100Hz. Gorassini, Eken, Bennett, Kiehn and Hultborn (2000) and Hennig and Lømo (1985) show that the firing rate in mice soleus muscles in vivo during activity ranges from 12Hz to 45Hz, but to reach maximal PF in vitro approximately 100Hz is needed. Another reason may be due to the fact that a soleus muscle consists a certain amount of type II muscle fibers (96.1%±2.9 type I) (Soukup, Zacharová, & Smerdu, 2002), depending on the type of mouse. Consequently, these type II fibers can still increase in PF when the muscle is stimulated at higher frequencies. As expected, PF did not increase above 100Hz (Vassilakos, James, & Cox, 2009; Haan de, 1998). The reason for using 50Hz-stimulation is that it mimics the firing rate of the soleus muscle of mice in vivo, as is mentioned above. During movements, James,

Altringham and Goldspink (1995) show that the soleus muscle is also active in near-isometric phases of movement. When a mouse moves, the complexity of activation patterns on the soleus muscle is far more than the activation pattern in vitro, they stated.

In contrast to PF, RFD increased further after 100Hz. This in in line with a study that demonstrates that reaching maximal RFD occurred at a higher frequency than maximal PF did (Haan de, 1998). An explanation for this is given by Vassilakos et al. (2009). He states that at higher stimulation frequency, Ca^{2+} release increases and that this release results in a higher RFD. He links this to in vivo conditions which state that during sprinting, a higher motor unit firing rate would be used compared to endurance activities to maximize RFD. Further research needs to be done to explain these results. This should contain a deeper analysis of physiological fatiguing processes at different stimulation frequencies and its influence on RFD.

5.3 Fatigue

The results of this investigation showed that when a soleus muscle is fatigued, PF and RFD decreased. However, the RFD decrease for all lengths combined only lasted until 6.5 minutes into the fatiguing protocol, in contrast to PF decrease for all lengths combined, which lasted throughout the whole fatiguing protocol. The magnitude of decline in PF and RFD (combined for all lengths) during fatigue were not statistically tested to each other, but when the relative values of both parameters are compared to each other after 10 minutes of fatiguing stimulation, it can be noticed that PF had declined to 41-67% and RFD to 36-59% of initial value. When relative values were compared halfway through the fatiguing protocol (i.e. 5min), PF had declined to 56-79% and RFD to 43-68% of initial value. This may indicate that RFD declines faster in the early phase of fatigue than PF does (Fig. 9 versus Fig. 11). The differences in magnitude of decline between PF and RFD are less well documented in the literature. An explanation for the differences in PF-RFD duration and magnitude in this study is that during the first two phases of fatigue, P_i accumulates and P_{Cr} decreases in the muscle (Allen, 2009). These changes have a physiological inhibitory effect on the cross-bridge cycling from its weak-bound to its strong-bound state, rather than influencing the number of cross-bridges itself. It is believed that this transition is the main factor that determines RFD (Fitts, 2008), so this could explain the possible differences between PF and RFD decline in the early phases of fatigue. Following this line of thought, the results of RT reinforce these observations. If PF and RFD would decline at the same rate, RT would remain constant (RFD: ΔForce/ΔTime, with Δ

representing 'change'). But results showed that RT increases during the first part of the fatiguing protocol, indicating that RFD declined more rapidly. The reason that PF decline lasts throughout the whole process, when all lengths are combined, of fatigue is because in the last phase of fatigue Ca^{2+} sensitivity and release are impaired and reduced. These changes cause a decline in number of activated cross-bridges and this is the main reason for a decrease in PF in the third phase of fatigue (Lännergren et al., 2006).

Comparison of different lengths, for both PF and RFD, resulted in statistical differences between muscle lengths. Which lengths were different in comparison to each other was not statistically tested (seen as a limitation in 5.5). Graphical analysis showed a greater relative (%) decline due to fatigue at optimal length and short lengths (L_0, L_{0-1} and L_{0-2}) than at longer muscle lengths (L_{0+1}, L_{0+2}) (Fig. 9 and Fig. 11). The reason PF seems to decline less at longer muscle lengths might be due to what Macintosh (2017) calls the length-dependent activation: when muscle/sarcomere length increases, Ca^{2+} sensitivity is increased, because the myofilaments will come closer to each other and this enhances the possibility of actin and myosin to interact/bind. As Ca^{2+} sensitivity increases with increase of muscle length, PF decline due to Ca^{2+} related fatigue will not be as great as PF decline at optimal length or short lengths. Greater PF decline at shorter lengths (i.e. short and optimal muscle length) due to fatigue in this study, is in contrast to what Lee et al. (2007) and Fitch and McComas (1985) found (namely greater fatigue in long muscle lengths – see introduction). MacNaughton and Macintosh (2006) stated that the measurement of passive force before contraction is not related to the passive force during a contraction. They suggest an alternative calculation, which is also used in this study: subtract the passive force associated with fascicle length when PF is reached. By doing so, during the execution of their in vitro study on gastrocnemius muscle of mice, Macintosh (2017) points out that there is still a length-dependence of fatigue: fatigue results in a downward shift of active force and the percentage of force decline is greater at short muscle lengths. In support of this finding, MacNaughton, Campbell, and MacIntosh (2007) inhibited Ca^{2+} release, mimicking fatigue, by introducing dantrolene (drug) in the muscle and demonstrate that a greater decrease of active force at shorter lengths (i.e. short and optimal muscle length) occurs in comparison to long muscle lengths. This can be attributed to the fact that interfilament distance is greater at shorter lengths and actin-myosin binding is more difficult according to Macintosh (2017).

The same results seemed to be found for PF were also found for RFD when different lengths were compared during fatigue. A possible explanation for the slower rate of fatigue in long muscle lengths is that there is less overlap of actin and myosin at long muscle lengths. If the overlap is less, less cross-bridges can be formed. This does not influence the RFD directly, but less cross-bridges means less ATP that gets hydrolyzed and thus less accumulation of P_i an P_{Cr} (i.e. less physiological fatigue). The muscles at short lengths seemingly fatigue at the same rate than the muscles at optimal length. Sacco, McIntyre, and Jones (1994) demonstrate that metabolic concentrations changes are the same after fatiguing stimulation of tibialis anterior muscle at 50Hz for optimal length and for short length. This can explain why RFD seemingly declined at the same rate for short muscle length and optimal muscle length. Further investigations on this topic are recommended and should focus on the change in metabolic rate and concentrations during fatiguing stimulation at optimal and short muscle lengths in vitro.

Recovery of RFD and PF, although not the main purpose of the study, showed an increase after 5 and 10 minutes in general when compared to the end of the fatiguing protocol. The muscles did not reach initial values after 5 or 10 minutes of recuperation for both PF and RFD. Graphical estimation seemed to show a mean recovery up to 80% of initial value for PF and 70% of initial value for RFD for all lengths combined. Derave, Op 't Eijnde, Ramaekers and Hespel (2005) found comparable results after fatiguing soleus muscle fibers of different mice types with six 2-minute bouts of repeated tetani (350ms duration and 50Hz frequency) with decreasing rest-intervals (3.8, 3.1, 2.6, 2.1, 1.6 and 1.3s). Force recovery (%) was measured after 5 and 10 minutes by a single tetanus. Soleus relative force does only recover to 70-80% of initial value, which is comparable with the results in our study. A possible explanation for this is that the muscles needed more than 10 minutes to recover from the fatiguing protocol, or that muscle damage occurred during the protocol.

5.4 Relevance to in vivo conditions: animal vs. human
PF is used nowadays to compare the injured limb to the contralateral healthy limb or to pre-injury PF values, because a drop in PF that results from this fatigue-induced decrease in cross-bridge formation can lead to a decrease in muscular performance and make a patient or athlete more susceptible to injuries. In that way, full recovery and readiness for return to sport

following an injury (for example an ACL reconstruction) can be seen as the ability to achieve 85% or 90% of the maximal force of the contralateral healthy limb or pre-injury levels (Angelozzi et al., 2012). But recent findings show that RFD could even be a more relevant parameter for making clinical decisions (Maffiuletti et al., 2016) as it is determined by the capacity of force generation in the early rising phase of a contraction and describes the ability of a skeletal muscle to rapidly develop force (Andersen & Aagaard, 2006). For this reason, Maffiuletti et al. (2016) proposes that RFD is better related to both sport-specific and daily movements: it shows the rate at which the intended force can be reached in order to react and move consequent during a performance. RFD can be evaluated to characterize explosive strength of individuals, such as patients, elderly and athletes (Maffiuletti et al., 2016). A correct interpretation of RFD during an isometric contraction is important and useful for assessing postural balance in elderly (Aagaard, 2003) or can be an outcome measure for return to sport decisions (Angelozzi et al., 2012). Optimal knowledge of RFD may be helpful in the development of interventions to increase explosive force production in athletes, ameliorate physical function, reduce fall risk in elderly and reduce injury in patients (Andersen & Aagaard, 2006). This can help coaches to identify optimal body positions (i.e. with optimal muscle lengths) and develop a specific training program to improve force production and delay the changes in force due to fatigue (Amasay, 2008).

5.5 Strengths and limitations

This study used a clearly defined protocol to induce fatigue in a muscle to investigate the possible influence of length. The in vitro setup allowed us to work very specifically at muscle level without influence of aspects, such as blood flow, nervous system etc. Another strength of this in vitro is that the settings, used in the protocol, could be easily controlled, such as stimulation frequencies, muscle lengths, …

Limitations included a small sample size of 40 soleus mice muscles. Each muscle length category that was tested, only included 8 muscles, which is too small to reach a high generalizability. Also, the impossibility of investigating the physiological events, such as Ca^{2+} release or P_i accumulation, limited the explanations that could be linked to the results of PF, RFD and RT. As this in vitro study focused on the muscle itself, fatigue had to be induced in a peripheral way via electrical stimulation. In this way, central fatigue due to restrictions in

nerve conduction during fatigue could not be taken into account. A major limitation could be the following: statistical differences in relative decline (both PF and RFD) between muscle lengths were assessed, but the inability of the researchers to compare each length separately to each other in order to statistically distinguish long muscle lengths (L_{0+1}, L_{0+2}) from short and optimal muscle lengths (L_{0-1}, L_{0-2}, L_0), led the researchers to perform a graphical analysis on this matter. By performing this graphical analysis, differences between short/optimal lengths and long lengths could be assessed, but lack of precise statistical comparison per length was a limitation that has to be recognized. Further investigations in a statistical manner in this domain have to be performed in the future. No experience of the researchers in this domain could influence the process of this study.

6. Conclusion

This study showed that muscle length influences force production, rate of fatigue and RFD. Fatigue was different between muscle lengths and showed to be greater at optimal and short muscle lengths than it is at longer muscle lengths (via graphical analysis), although further and precise assessment is necessary. This can be important to identify optimal body positions during sport and develop optimal training programs for specific sports. This study also showed that RFD probably declines faster during fatigue than PF does. This can be clinically important to observe fatigue in early stages. Further research should be done with bigger sample sizes and different muscle fiber types so results could be compared.

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8. Appendix

Figure 14: Inventory form master thesis part 2

       Figure 14.1: Inventory form master thesis part 2 – Lennert Mooren

       Figure 14.2: Inventory form master thesis part 2 – Lennart Ghijsens

INVENTARISATIEFORMULIER WETENSCHAPPELIJKE STAGE DEEL 2

DATUM	INHOUD OVERLEG	HANDTEKENINGEN	
18/09/'18	Start inleiding + materiaal en methode overleg. Uitleg metingen in vitro / computeranalyse	Promotor: Pieter van Noten Copromotor/Begeleider: / Student(e): Lennart Mooren Student(e): Lennart Ghijsens	✓
25/09/'18	Overleg gevonden resultaten omtrent includeren in inleiding. Verdere aanwijzingen i.v.m. met methode	Promotor: Pieter van Noten Copromotor/Begeleider: / Student(e): Lennart Mooren Student(e): Lennart Ghijsens	✓
24/01/'19	Uitleg data-analyse Computer	Promotor: Pieter van Noten Copromotor/Begeleider: / Student(e): Lennart Ghijsens Student(e):	✓
31/01/'19	Uitleg data-analyse Computer	Promotor: / Copromotor/Begeleider: Jan Spaas Student(e): Lennart Mooren Student(e): /	✓
28/03/'19	Overleg resultaten data-analyse en verdere uiteenzetting uitschrijven thesis.	Promotor: Pieter van Noten Copromotor/Begeleider: / Student(e): Lennart Mooren Student(e): Lennart Ghijsens	✓
24/04/'19 30/04/'19 1/05/'19 7/05/'19	Uitleg statistische verwerking resultaten + samenkomen voor overleg en verdere aanwijzingen	Promotor: Pieter van Noten Copromotor/Begeleider: / Student(e): Lennart Ghijsens Student(e):	✓
		Promotor: Copromotor/Begeleider: Student(e): Student(e):	
		Promotor: Copromotor/Begeleider: Student(e): Student(e):	
		Promotor: Copromotor/Begeleider: Student(e): Student(e):	
		Promotor: Copromotor/Begeleider: Student(e): Student(e):	

Figure 14: Inventory form master thesis part 2.

In te vullen door de promotor(en) en eventuele copromotor aan het einde van MP2:

Naam Student(e): ...MOOREN LENNERT........................ Datum: 30/05/2019

Titel Masterproef: Does muscle length effect fatigue in soleus muscle of mice?

1) Geef aan in hoeverre de student(e) onderstaande competenties zelfstandig uitvoerde:
   - NVT: De student(e) leverde hierin geen bijdrage, aangezien hij/zij in een reeds lopende studie meewerkte.
   - 1: De student(e) was niet zelfstandig en sterk afhankelijk van medestudent(e) of promotor en teamleden bij de uitwerking en uitvoering.
   - 2: De student(e) had veel hulp en ondersteuning nodig bij de uitwerking en uitvoering.
   - 3: De student(e) was redelijk zelfstandig bij de uitwerking en uitvoering
   - 4: De student(e) had weinig tot geringe hulp nodig bij de uitwerking en uitvoering.
   - 5: De student(e) werkte zeer zelfstandig en had slechts zeer sporadisch hulp en bijsturing nodig van de promotor of zijn team bij de uitwerking en uitvoering.

Competenties	NVT	1	2	3	4	5
Opstelling onderzoeksvraag	⊗	O	O	O	O	O
Methodologische uitwerking	⊠	O	O	O	O	O
Data acquisitie	O	O	O	O	⊠	O
Data management	O	O	O	O	⊠	O
Dataverwerking/Statistiek	O	O	O	O	⊠	O
Rapportage	O	O	O	O	⊠	O

2) **Niet-bindend advies:** Student(e) krijgt toelating/~~geen toelating~~ (schrappen wat niet past) om bovenvermelde Wetenschappelijke stage/masterproef deel 2 te verdedigen in bovenvermelde periode. Deze eventuele toelating houdt geen garantie in dat de student geslaagd is voor dit opleidingsonderdeel.

3) Deze wetenschappelijke stage/masterproef deel 2 mag wel/~~niet~~ (schrappen wat niet past) openbaar verdedigd worden.

4) Deze wetenschappelijke stage/masterproef deel 2 mag wel/~~niet~~ (schrappen wat niet past) opgenomen worden in de bibliotheek en docserver van de UHasselt.

Datum en handtekening Student(e)	Datum en handtekening promotor(en)	Datum en handtekening Co-promotor(en)
31/05/2019	31/05/2019	

Figure 14.1: Inventory form master thesis part 2 – Lennert Mooren

In te vullen door de promotor(en) en eventuele copromotor aan het einde van MP2:

Naam Student(e): _GHIJSENS LENNART_ Datum: _30/05/2019_

Titel Masterproef: _Does muscle length effect fatigue in soleus muscle of mice?_

1) Geef aan in hoeverre de student(e) onderstaande competenties zelfstandig uitvoerde:
   - NVT: De student(e) leverde hierin geen bijdrage, aangezien hij/zij in een reeds lopende studie meewerkte.
   - 1: De student(e) was niet zelfstandig en sterk afhankelijk van medestudent(e) of promotor en teamleden bij de uitwerking en uitvoering.
   - 2: De student(e) had veel hulp en ondersteuning nodig bij de uitwerking en uitvoering.
   - 3: De student(e) was redelijk zelfstandig bij de uitwerking en uitvoering
   - 4: De student(e) had weinig tot geringe hulp nodig bij de uitwerking en uitvoering.
   - 5: De student(e) werkte zeer zelfstandig en had slechts zeer sporadisch hulp en bijsturing nodig van de promotor of zijn team bij de uitwerking en uitvoering.

Competenties	NVT	1	2	3	4	5
Opstelling onderzoeksvraag	⊘	O	O	O	O	O
Methodologische uitwerking	⊘	O	O	O	O	O
Data acquisitie	O	O	O	O	⊘	O
Data management	O	O	O	O	⊘	O
Dataverwerking/Statistiek	O	O	O	O	⊘	O
Rapportage	O	O	O	O	⊘	O

2) **Niet-bindend advies:** Student(e) krijgt toelating/~~geen toelating~~ (schrappen wat niet past) om bovenvermelde Wetenschappelijke stage/masterproef deel 2 te verdedigen in bovenvermelde periode. Deze eventuele toelating houdt geen garantie in dat de student geslaagd is voor dit opleidingsonderdeel.

3) Deze wetenschappelijke stage/masterproef deel 2 mag wel/~~niet~~ (schrappen wat niet past) openbaar verdedigd worden.

4) Deze wetenschappelijke stage/masterproef deel 2 mag wel/~~niet~~ (schrappen wat niet past) opgenomen worden in de bibliotheek en docserver van de UHasselt.

Datum en handtekening Student(e)
31/05,

Datum en handtekening promotor(en)
37/05/2019

Datum en handtekening Co-promotor(en)

Figure 14.2: Inventory form master thesis part 2 – Lennart Ghijsens