NORGES IDRETTSHØGSKOLE

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Sex differences in muscle function and myocellular response to a strenuous military field exercise

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Abstract

Purpose: The purpose of this study was to investigate sex differences in the physiological and myocellular responses to a strenuous military field exercise (FEX), both acutely and in the recovery phase.

Methods: Soldiers (F=8, M=10) from the Norwegian Defense Cyber Academy conducted a 10-day strenuous FEX. Body composition was measured by bioelectrical impedance before (T1) and 0 (T2-0), 1 (T2), 7 (T3) and 14 (T4) days after the FEX, whereas as maximal leg extension force was tested at T1, T2, T3 and T4. Skeletal muscle biopsies were obtained from *m. vastus lateralis* at T1, T2 and T3. Biopsies were analyzed for the following proteins involved in regulation of protein balance and metabolism by western blots: 4E-BP1, p70S6K, AMPKα, Ulk1, COX-IV, HADH, CS and Na^+K^+β1.

Results: Lean leg mass increased with 4.4 ± 2.6 % for men and women combined at T2-0 and returned to pre values at T3, with no sex difference. Men and women combined declined in maximal leg extension force with 9.2 ± 11% at T2 ($p<0.05$) and was still 8.8 ± 13% reduced at T4 ($p<0.05$), with no sex differences. The ratio between phosphorylated and total AMPK ($AMPK_{P/T}$) increased by 39 ± 40% at T2 ($p<0.05$) and by 48 ± 47% at T3 ($p<0.001$). At T3, we also report a 46 ± 49% increase in the phosphorylation of $AMPK^{Thr172}$ ($p<0.01$). We observed tendencies for sex difference in the relative change from T1 for 4E-BP1 ($p=0.082$) and Na^+K^+β1 ($p=0.065$) at T2 and in p70S6K ($p=0.057$) at T3. At T2, there were a significant different in the change in Ulk1 ($p<0.05$), where women decreased 22 ±15 % and men increased 16 ± 17%. Na+K+β1 decreased by 7 ± 12% ($p<0.05$) at T2 and 12 ± 13% ($p<0.05$) at T3. COX-IV decreased 15 ± 43% at T2 ($p<0.05$), and was recovered at T3, whereas CS decreased 18 ± 21% ($p<0.05$) at T3. There were no significant changes in HADH.

Conclusion: A strenuous military field exercise resulted in similar reductions in maximal force for men and women together with increased catabolism and/or inhibited anabolism, which lasted for at least one week. There was a sex difference in the change in protein levels of Ulk1, but this was not related to any difference in reduction of maximal force. The results indicate that there may be sex differences in the myocellular response to a military field exercise, but further investigations are needed.

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Table of contents

Acronyms

AMPK - 5' AMP-activated protein kinase

ATP – Adenosine triphosphate

BIA – Bioelectrical impedance analysis

(Muscle) CSA – Muscle cross-sectional area

CK – Creatine kinase

CMJ – Countermovement jump

COX-IV - Cytochrome c oxidase or complex IV

CS - Citrate Synthase

ECC – Excitation-contraction-coupling complex

FEX – Field exercise

FFM – Fat-free mass

FIH - Norwegian Defense University College of Engineering

FM - Fat mass

h - hours

HADH - Hydroxyacyl CoA dehydrogenase

IGF-1 - Insulin-like growth factor 1

kDa – Kilodalton

LBM - Lean body mass

LC3b - light chain 3 beta

mTORC1 - mechanistic target of rapamycin complex 1

MU - motor unit

MVC – Maximum voluntary contractions

P/T – Phosphorylated/Total protein

p70S6K1 - ribosomal protein of 70-kDa S6 kinase 1

RFD - Rate of force development

RyR – Ryanodine receptor

SF – Special Forces

SR – Sarcoplasmic reticulum

TCA - Tricarboxylic acid cycle

Ulk1 - unc-51 like autophagy activating kinase 1

USRT - U.S. Ranger Training

1RM - One repetition maximum

4E-BP1 - 4E-binding protein 1

1. Introduction

Having a high level of physical fitness is necessary for successful military performance, because the physical demands of an operational task can be very high (Hydren, Borges & Sharp, 2017). The training regime for soldiers serving in Special Forces (SF), is even thought to be more demanding than what is undertaken by elite athletes (Hoffman et al., 2016). Military operations may further expose soldiers to extreme stress, including severe energy deficit, sleep deprivation and physical strain (O'Hara, Henry, Serres, Russel & Locke, 2014), which is why strenuous military training and field exercises are used to prepare the soldiers. A strenuous military field exercise (FEX) normally vary from long periods of low intensity to short periods of very high intensity, leading to very high energy expenditure. Together with low energy intake during these periods, this can result in energy deficits of 40% or more (Margolis et al., 2014). These exercises often also consist of lack of sleep and other environmental and psychological challenges. Consequences of these types of training exercises are reductions in body mass, lean body mass (LBM) and fat mass (FM), as well as reductions of physical performance (Nindl, Friedl, Frykman, Marchitelli, Shippee & Patton, 1997; Shippee, Askew, Bernton, Martinez-Lopez & Kramer, 1994). It is logical to believe that the changes in body composition, especially in LBM, is the main reason for the reduction in performance, because LBM is a main determinant of both maximal muscle force (Maffiuletti, Aagaard, Blazevich, Folland, Tillin & Duchateau, 2016) and anaerobic performance (Raastad, Paulsen, Refsnes, Rønnestad & Wisnes, 2010; Vikmoen, 2015). However, the reduction in the soldiers' physical performance may be severely impaired for several weeks after a strenuous FEX, whereas the body mass and composition seem to recover within one week of rest (Hamarsland, Paulsen, Solberg, Slaathaug & Raastad, 2018; Raustøl, 2018). This suggests that the reduced performance after such exercises cannot solely be explained by changes in muscle mass, and that other physiological or muscular changes that may occur potentially takes longer to recover. Current observations indicate that a strenuous FEX causes a negative protein balance that is primarily affected by increased protein breakdown (Margolis et al., 2014; Moberg et al., 2017). Negative energy balance in muscle tissue is a signal to degrade proteins in order to provide energy and amino acids to other tissue (Frayn, 2010), which may be the reason for the observed increase in protein breakdown. A second reason could be intensified myocellular need to degrade or remove damaged or dysfunctional proteins (Ferrier, 2014). However, the physiological mechanisms behind the long-term impaired performance are nevertheless very little surveyed and the interactions between the changes

that occur after demanding FEX are complex. Therefore, the precise mechanisms behind altered body composition and reduced performance are unclear (Friedl, 1995; Hamarsland et al., 2018; Hoyt, Opstad, Haugen, DeLany, Cymerman & Friedl, 2006; Raustøl, 2018). Knowledge of these changes is crucial to being able to implement effective measures to minimize the negative effects a FEX have on performance, but also to find the most effective recovery strategy in order to ensure fully operational soldiers. A negative protein balance could, in example, be counteracted with increased protein intake (Phillips, 2009). A part from two studies measuring the acute recovery three hours after a FEX (Christensen et al., 2008; Thorlund et al., 2011), there are, to our knowledge, only three studies reporting on the physical recovery of strenuous military training (Hamarsland et al., 2018; Nindl et al., 1997; Raustøl, 2018) and for the myocellular mechanisms in the recovery phase there are none. To investigate these mechanisms, more complex physical tests must be performed while taking muscle biopsies to examine the changes and recovery of the muscle cells.

The majority of military studies have been conducted solely on men. Even so, the number of women serving and enlisting has been increasing over the recent years and will probably continue to do so. In most aspects of physical performance men perform better than women (Bækken & Teien, 2016; Hunter, 2016). However, there are indications that both men and women decrease similar in relative physical performance even if the men experience a greater loss of fat-free mass (FFM) after FEX, and that during the recovery phase the women return faster than men to baseline values in FFM and physical performance (Hoyt et al., 2006; Raustøl, 2018). Theoretically, a faster recovery in women compared to men is thought to occur because women have lower resting metabolism, they are better able to tolerate changes in body composition, and they utilize fat reserves better which promotes protection against LBM losses (Friedl, 1995; Hamarsland et al., 2018; Pasiakos & Margolis, 2017; Tassone & Baker, 2017). To our knowledge only one study have investigated sex differences in how physical performance are affected by and recover from a demanding FEX (Raustøl, 2018), and there are no studies on possible sex differences in the myocellular recovery after a FEX.

1.1 Aim

Investigating sex differences during and in the recovery phase of FEX will contribute to a better understanding of how both men and women are physiologically affected by these exercises. This will be important knowledge for a successful implementation of more women in the Armed Forces. Therefore, the aim of this study was to investigate sex differences in the

response in LBM and force-generating capacity, and myocellular responses involved in the regulation of protein balance and energy production, to a strenuous FEX, both acutely and in the recovery phase. We hypothesized that the responses to a FEX would be similar for the sexes, and that women would show a faster recovery compared to men. Furthermore, we hypothesized that regulators of protein balance would shift to a more negative state right after the FEX, and that it would increase during the recovery phase.

2. Theory

2.1 Muscle force

Muscle force is defined as the maximal force or torque a muscle or muscle-group can produce by a specific or predetermined velocity (Raastad et al., 2010). Maximal voluntary muscle force (MVC) is the maximal force a muscle or muscle group can produce under slow contractions or isometric muscle actions (Raastad et al., 2010). The muscle force produced when the velocity of a movement reaches zero, is defined as the isometric strength (Peterson, Alvar & Rhea, 2006). Isokinetic strength is a muscle force applied in muscle actions where the joint angular velocity is constant through the movement (Raastad et al., 2010).

Explosive strength is the ability to produce the greatest possible force as quickly as possible, and is most commonly described by the power product (force · velocity) (Raastad et al., 2010). Muscular peak power is the maximum product of force and speed obtained during a given movement (Peterson et al., 2006). The rate of force development (RFD) is another important measurement for explosive strength, which describes the force-time curves during contractions (Maffiuletti et al., 2016).

Muscular endurance is the ability of the muscle or muscle group to sustain repeated contractions against submaximal resistance for an extended period of time (Epstein, Yanovich, Moran & Heled, 2013; Raastad et al., 2010).

2.1.1 Factors affecting muscle force production

Force production is a product of the relationship between muscular properties, like architecture and muscle size, and neural factors, like motor unit (MU) recruitment and discharge rate (Maffiuletti et al., 2016). The main contributing factors vary depending on the force-velocity relationship involved is the specific movement (Raastad et al., 2010). Considering muscular factors, in example, the most important factor for slow contractions is the cross-sectional areal (CSA). For fast contractions, on the other hand, the fiber type composition and muscle length is more important (Raastad et al., 2010).

The muscle CSA is determined by the amount and size of each muscle fiber, which is further determined by the total number of sarcomeres in parallel. The total number of sarcomeres determines the available amount of cross-bridge cycles to be activated and consequently the development of force (Raastad et al., 2010). This explains why muscle size is a main determinant of MVC (Maffiuletti et al., 2016). Large muscles are also favorable in exhibiting

a higher RFD, because of a greater extent of parallel elastic material as wells as a greater absolute force (Maffiuletti et al., 2016).

Muscle fiber type is a major influencer of contractile properties of the muscles, because the different fiber types have different properties (Raastad et al., 2010). Fast twitch fibers (type 2) are, in example, able to produce higher force at faster shortening velocities, compared to slow twitch fibers (type 1), which allows type 2 to produce more power at a given shortening velocity (Raastad et al., 2010). Type 2 also has a faster rate of tension, which especially influences the RFD (Maffiuletti et al., 2016). The reasons for the differences could be that type 2 has greater total sarcoplasmic reticulum (SR) Ca^{2+}-release per action potential, faster time constants of Ca^{2+}-currents and faster cross-bridge cycling rates, than type 1. The greater Ca^{2+}-release from SR in type 2 may further be explained by a 200 % greater ryanodine receptor (RyR) content, larger number of junctional t-tubular segments and greater total SR development. Type 2 also have 200-300% greater Na^{+} channel density than type 1, and may therefore be better able to conduct high rates of excitatory potentials resulting from high discharge rates (Maffiuletti et al., 2016). Type 1 fibers are, on the other hand, more resistant to fatigue and more efficient (Coyle, 1995; Nindl, Jones, Van Arsdale, Kelly & Kraemer, 2016).

The muscle architecture affects how quickly and effectively the force is transmitted to the tendon and how fast the muscle may contract. Increases in fascicle angle allow for a greater physiological CSA for a given muscle size, and thus for a greater force. Forces are, on the other hand, more directly transmitted to the tendon in muscles with lesser fascicle angle, which is preferred for fast contractions (Maffiuletti et al., 2016; Raastad et al., 2010). Longer muscle fibers, in the longitudinal direction of the muscle, are able to develop greater force during fast contractions. Because of a greater extent of series elastic material they may on the other hand exhibit a slower RFD (Maffiuletti et al., 2016; Raastad et al., 2010). Force transmission is also affected by the musculotendious stiffness, and tissue stiffness is inversely proportional to length. With longer tissues, the compliancy is greater and the force transmission slower (Maffiuletti et al., 2016).

Voluntary RFD in the initial phase of a contraction may be strongly affected by the speed-related properties of the muscle and MVC if the contraction is slow. The ability to produce force rapidly is, on the other side, mostly dependent on the increase of muscle activation at the onset of the contraction. The magnitude of this activation depends on the amount of active

motor units (MU) and the rates at which motor neurons discharge action potentials (Maffiuletti et al., 2016). The relative contributions of the two vary with contraction speed. Slow contractions have a progressive activation of MUs, while fast contractions recruits MUs at much lower forces. In most muscle, both recruit MUs to an upper limit corresponding to approximately about 80-90% of the maximum force To increase the muscle force beyond this limit depends entirely of an increased discharge rate. Slow contractions increase the discharge rate of MUs progressively, while fast contractions exert a high initial discharge rate that declines progressively with consecutive discharges (Maffiuletti et al., 2016).

2.1.2 Measuring muscle force

Several methods are available to measure muscle force, but all have different strengths and weaknesses in regards to the training principles and scientific implementation. Isometric contractions, in example, produce maximal force, but this test is not in accordance with the principle of specificity in training (Peterson et al., 2006). Testing strength with an isokinetic dynamometer could be very useful because specific muscle groups can be targeted, but the constant velocity of the muscular contraction does not occur during normal muscular activity and it can be difficult to conduct in a field-testing, particularly with time constraints (Friedl, 1995). The one repetition maximum (1RM) test, for any specific exercise, measures the maximum weight that can be lifted through an eccentric and concentric range of motion (Peterson et al., 2006). The 1RM is, in fact, one of the most common methods for testing strength and is considered a gold standard (Peterson et al., 2006; Raastad et al., 2010). It has also been established a linear relationship between lower body muscular force and power (Peterson et al., 2006). This contributes to more flexibility when deciding which test to use. One possibility is jump tests, which is a commonly used method to measure muscle force and power in the lower extremities (Raastad et al., 2010). These test are probably also more suitable for field-testing compared to a 1RM test, as they are easy to conduct and require little equipment. Muscular endurance tests could potentially also be easy to execute for field-testing, as it may be examined through the maximal number of repetitions completed with submaximal resistance. No matter which test that is chosen, it should be relevant for the activity and it needs to be reliable (Raastad et al., 2010).

2.2 Physical demands to a military soldier

2.2.1 Operational tasks

The physical requirements for the operational tasks a soldier can be exposed to are widely

varied (Hoffman et al., 2016; Hydren et al., 2017). These tasks may, as well, be somewhat unpredictable since they are affected by what the opposing forces will do. Most of the objectives will nonetheless include variations of different physical demands that are crucial to the soldier's operability. This may include wall climbs, marching or running with heavy loads, dragging or carrying wounded to safety, crawling, lifting or jumping, to mention some (Hoffman et al., 2016). As much as 73% of the physical military tasks, a soldier executes, seem to rely on strength performances. A report by the U.S. Navy actually claimed that, of all the physically demanding jobs, 84% consisted of lifting, carrying and pulling (Hydren et al., 2017). These tasks highlight the importance of strength and power development for combat personnel, which are also strongly related to high-intensity military tasks (Mala, Szivak, Flanagan, Comstock, Laferrier, Maresh & Kraemer, 2015). For soldiers serving in SF, it has been emphasized that their training is more demanding than what is undertaken by elite athletes (Hoffman et al., 2016). Soldiers like those in SF, are very physically fit, and has shown comparable power outputs to collegiate athletes as well as markedly excelling in muscle strength compared to the average soldier (Nindl et al., 1997).

2.2.2 Muscular strength and military performance

Physical performance tests are commonly used to predict occupational task performance in order to evaluate the ability of individuals to do a job (Hydren et al., 2017). The muscular factors described earlier contributes in somewhat different manners in soldiers performance, depending on which task they are performing. The maximum voluntary force (MVC) production comes in handy during task consisting of slow contractions, like marching with heavy loads or carrying wounded personnel. Other task involve more explosive actions, like sprinting from cover to cover, avoiding bullets or jumping, and are dependent more on explosive strength.

1RM is, as mentioned, the test most commonly used to measure muscle strength. The 1RM box lift has, in particular, been used to investigate strength for heavy lifting jobs (Hydren et al., 2017). This test has shown the highest correlation with field performance ratings of light infantry soldiers and even if this test measures the MVC, it is well correlated to other tests of muscular strength (Friedl, 1995; Shippee et al., 1994). The 1RM box lift is easy and safe to execute, but this test may not necessarily be the best test for field-testing (Hydren et al., 2017). Different fitness-related tests have therefore been investigated to see which correlates most and predict performance of the maximal lifting capacity. The strongest predictor for 1RM box lift test seems to be jumping performance, both horizontally and vertically (Hydren

et al., 2017). This is not surprising, given that maximal strength capacity is significantly correlated with voluntary RFD. RFD is also viewed as more sensitive to detect acute and chronic changes in neuromuscular function, which seems to be better related to most performances of both sport-specific and functional daily tasks (Maffiuletti et al., 2016).

Dynamic 1RM tests for other muscle groups, like shoulder press or bench press, may also be strong predictors for maximal lift capacity, with upper and full body measurements showing good-to-excellent correlations (Hydren et al., 2017). Isometric strength may be another good predictor to evaluate larger muscle groups. Using muscular endurance tests as, on the other hand, is not preferable as it only shows fair-to-moderate correlations to maximal lifting capacity, and with poor reliability (Hydren et al., 2017). It may exist some sex differences in which tests are the best predictors, though. 1RM box lift and sit-ups, and 1RM and maximum lift mean power, seem to have a higher correlation for men than for women, whereas 1RM and isometric leg extensor strength has a higher correlation for women than for men (Hydren et al., 2017).

2.3 Protein balance

The net protein balance is decided by the rate of protein synthesis and protein breakdown. For the protein synthesis the two hormones insulin and growth hormone has the general anabolic role. The rate of protein breakdown is generally controlled by the insulin, cortisol and thyroid hormones (Frayn, 2010). Nonetheless, there are also other influencers, like testosterone, physical activity, ingestion of food and adrenergic stimulation (Areta et al., 2014; Frayn, 2010; Moberg et al., 2017; Phillips, 2009). A balance in synthesis and breakdown is necessary to maintain the cell homeostasis. The relationship contributes to continuous replacement of contractile proteins and organelles, which is crucial for proper muscle function (Phillips, 2009; Sandri, 2013). As much as 300-400grams of the body proteins are thought to be replaced every day by simultaneous protein breakdown and synthesis (Ferrier, 2014). Having a positive protein balance over time will lead to muscle hypertrophy, as a negative protein balance over time will lead to muscle atrophy (Sandri, 2013). The following sections will shortly describe the mechanisms and regulations of the protein balance.

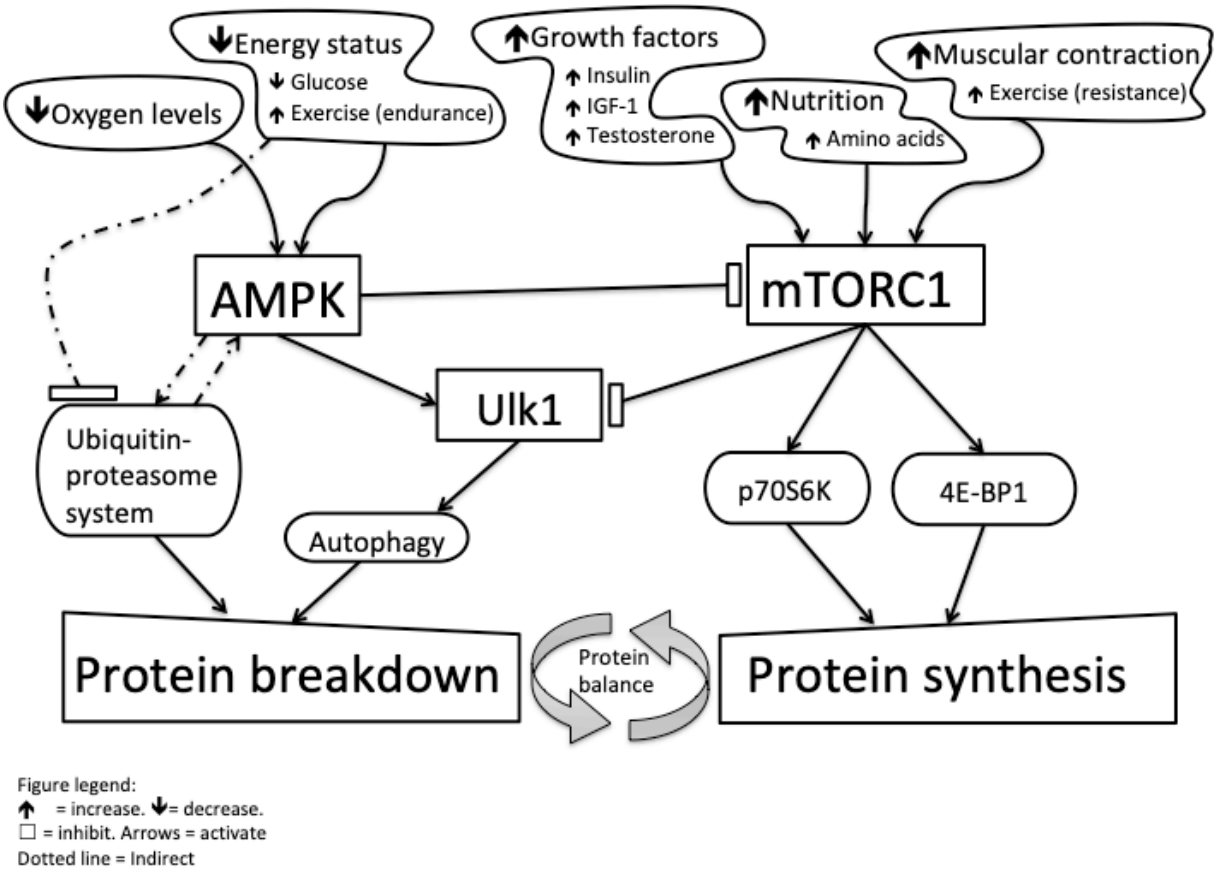


***Figure 1**: A simplified schematic overview of important regulators of the protein balance*

2.3.1 Protein synthesis

Genetic information is expressed through transcription to RNA and, in the case of messenger RNA (mRNA), subsequently into proteins by translation. This is where the mRNA language is translated onto the language of an amino acid sequence, which we normally refer to as protein synthesis. The translation happens in the ribosomes and is initiated in the cytosol by the assembly of the mRNA to be translated, the aminoacyl-tRNA specified by the first codon in the message, Guanosine-5'-triphosphate and initiation factors that facilitate the assembly of the initiation complex. Newly made proteins undergo a number of processes to achieve their functional form, and many are covalently modified to activate them or alter their activities (Ferrier, 2014).

The mechanistic target of rapamycin complex 1 (mTORC1) is the key-mediator of the protein synthesis, where mTOR is a protein kinase, which becomes active through alterations of the associations with other regulatory proteins (Frayn, 2010; Fyfe, Bishop, Bartlett, Hanson, Anderson, Garnham & Stepto, 2018; Moberg et al., 2017). mTORC1 modulates protein synthesis by regulating its downstream effectors, the eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1), and the ribosomal protein of 70-kDa S6 kinase 1 (p70S6K1)

(Jespersen et al., 2015; McGlory, Devries & Phillips, 2017; Pagano, Py, Bernardi, Candau & Sanchez, 2014).

4E-BP1 is a translation repressor protein that has several phosphorylation sites. When not phosphorylated at Ser65 and Thr70, it inhibits translation by binding to the translation initiation factor eIF4E. Hyper-phosphorylation of 4E-BP1 disrupts this interaction and results in activation of translation (Fadden, Haystead & Lawrence, 1997). mTORC1 phosphorylates other sites than Ser65 and Thr70, but is thought to prime 4E-BP1 for subsequent phosphorylation (Gingras et al., 1999).

The activity of p70S6K is controlled by multiple phosphorylation events, which helps the mitogen control cell growth and G1 cell cycle progression (Dufner & Thomas, 1999; Pullen & Thomas, 1997). When activated by mTORC1, it subsequently promotes ribosomal binding to mRNA to initiate protein synthesis (McGlory et al., 2017). The p70S6K kinase activity *in vivo* is most closely correlated with the phosphorylation of $p70S6K^{Thr389}$ (Weng, Kozlowski, Belham, Zhang, Comb & Avruch, 1998).

Activation of the translational machinery is generally thought to mediate the early rise in protein synthesis in response to anabolic stimuli. However, the cellular protein synthesis rates are not only determined by changes in the translational efficiency, but also by the translational capacity. This capacity may increase through ribosome biogenesis, if there is a prolonged anabolic stimulus over weeks or months (Fyfe et al., 2018). Catabolic stimuli would, on the other side, inhibit this long-term effect. Presence of energy deficit, reduced contractile activity or insufficient amounts of amino acids are such stimulus and can down regulate mTORC1 signaling which will further reduce the rate of protein synthesis (Moberg et al., 2017).

Single sessions of concurrent exercise have demonstrated no change in mTORC1-signalling or rates of muscle protein synthesis (Fyfe et al., 2018). However, the combination of endurance training and resistance training is thought to attenuate muscle hypertrophy adaptation. The reason for this hypothesis is that increased AMPK, acting to restore the energy balance, inhibit the activity of mTOR. A study investigating this hypothesis on training-accustomed individuals showed that the phosphorylation of p70S6K1 increased to a greater extent after eight weeks of training with resistance training alone, than combined with endurance training. This implies higher activity by mTORC1 and therefore higher muscle protein synthesis without endurance training (Fyfe et al., 2018). It should be noted, though,

that the ribosome biogenesis markers were favorable for the groups combining concurrent and resistance training.

2.3.2 Protein breakdown

The breakdown of proteins is an important process, making sure to degrade and remove damaged or dysfunctional proteins (Ferrier, 2014). Abundance of these types of proteins may over time cause muscle atrophy (Sandri, 2013). Protein breakdown is primarily evolved around two systems, the autophagy-lysosomal pathway and the ubiquitin-proteasome system (Moberg et al., 2017).

Autophagy

Autophagy is a process that is required for maintenance of muscle mass and muscle function and may be important for exercise adaptations (Schwalm et al., 2015). The initiation of this degradation process is controlled by the unc-51 like autophagy activating kinase 1 (Ulk1) protein complex (Moberg et al., 2017). The autophagy pathway specifically degrades obsolete parts of the cell, starting off by enclosure of an organelle by a double membrane, which creates an autophagosome (Alberts et al., 2013). The final formation of the autophagosome involves the lipidation of microtubule-associated protein1 light chain 3 beta (LC3b-I), which results in the formation of LC3b-II, which further attaches to the autophagosome membrane (Moberg et al., 2017). The autophagosome then fuses with a lysosome where the material can be degraded. The amino acids that are generated from this process may then be recycled into a continued protein synthesis (Alberts et al., 2013).

These processes usually increase in situations where the cell is starved or when it's remodeling in response to development (Alberts et al., 2013). In fact, The Ulk1 protein complex is activated when phosphorylated by AMP-activated protein kinase (AMPK) and inhibited when phosphorylated by mTORC1 (Moberg et al., 2017). Opposed to mTORC1, which acts to promote protein synthesis, AMPK is a sensor of cellular energy status and act by inhibiting anabolic cellular processes and stimulating catabolism (Fyfe et al., 2018; Pagano et al., 2014). This enzyme is activated by adenosine monophosphate (AMP), which increases in concentration during energy deficit, and inhibited by adenosine triphosphate (ATP) (Frayn, 2010; Hardie, 2004). During conditions with cellular energy depletion, AMPK is activated and directly phosphorylates Ulk1 at multiple sites (Egan et al., 2011; Kim, Kundu, Viollet & Guan, 2011; Pagano et al., 2014). When mTOR, which is a regulator of cell growth and an inhibitor of autophagy, is in it's active state it phosphorylates Ulk1 and disrupts the

interaction between Ulk1 and AMPK (Kim et al., 2011).

The autophagy flux can be measured using the ratio LC3b-II/LC3b-I and p62/SQSTM1-protein levels (p62), which is considered to be valid indicators for the autophagosome synthesis and clearance, respectively (Moberg et al., 2017; Pagano et al., 2014). Increased LC3b-II/LC3b-I ratio indicates increased synthesis of autophagosomes, whereas increased p62-protein levels indicates increased clearance (Moberg et al., 2017). For physical exercise lasting ≤ 2h, the ratio LC3b-II/LC3b-I seems to decline together with activation of upstream signaling (Moberg et al., 2017). For more extreme exercises, like ultra-marathons, the opposite has been reported (Jamart, Francaux, Millet, Deldicque, Frère & Féasson, 2012). Increases in the LC3b-II/LC3b-I ratio has also been observed during eight days of strenuous FEX, together with increased phosphorylation of the autophagy regulator Ulk1. However, no change was observed in p62-protein levels, which suggest a possible protein sparing mechanism through unchanged autophagosome clearance or a blockage of the autophagy flux (Moberg et al., 2017). The protein levels of p62 seem to depend on the exercise intensity, reducing protein levels only during high intensity (Schwalm et al., 2015).

Ubiquitin-proteasome

Proteasomes are present in both the cytosol and the nucleus. These are large protein machines that contain a cylinder formed from proteases. Their role is to bind proteins that are marked for degradation and to unfold them inside the cylinder, energized by ATP hydrolysis. The proteins destined to degradation are chopped into short peptides by proteases, which hydrolyze the peptide bonds between amino acids. The peptides are then released from either end of the proteasome cylinder (Alberts et al., 2013).

The protein that is responsible for allowing the proteasome complex bind to a protein is a small protein called ubiquitin. Ubiquitin marks the proteins, which causes them to bind to the end of proteasome cylinders where they are further pushed into the cylinder. The marked proteins will then be degraded, while the ubiquitin is recycled out into the cytosol again (Alberts et al., 2013). Ubiquitin is controlled by ubiquitin ligases, like Muscle atrophy F box (MAFbx or Atrogin-1) and muscle ring finger-1 (MuRF-1). The gene expression of particularly MuRF-1 has been illustrated to increase after both endurance and resistance exercise (Moberg et al., 2017). Activation of these two will lead to muscle atrophy via muscle protein breakdown (Jespersen et al., 2015).

2.4 Mitochondrial homeostasis

The mitochondria are the major sites for ATP production in the cell (figure 2), and contain enzymes active in fatty acid oxidation (FAO), tricarboxylic acid cycle (TCA) and oxidative phosphorylation (Widmaier, Raff & Strang, 2014). The mitochondrial content of the muscle is thought to be a major determinant of the degree of muscle stress during exercise, because it determines the amount of mitochondria that are available to share the respiration needed to produce enough ATP (Coyle, 1995). With a large number of mitochondria available to share the respiration, the need for respiration through each mitochondrion is relatively small. This means that during disturbances of myocellular homeostasis, the disturbances of the ATP production may be less affected (Coyle, 1995). A small number of mitochondria will, on the other hand, possibly lead to a larger myocellular disturbance and a faster development of fatigue. Even so, when muscular fatigue causes increased muscle glycogenolysis, glycolysis and lactate production, it also negatively affects the mitochondrial respiration to produce ATP. It is, nevertheless, suggested that a larger muscle mass has a larger amount of mitochondria, and that this reduces the requirement for ATP synthesis from each mitochondria (Vikmoen, 2015). The mitochondrial activity is not only, however, determined by the total amount of mitochondria and may be more dependent on the total oxidative capacity of each mitochondrion. The current belief is that the mitochondria only work at a submaximal rate at VO_{2max} and therefore that the mitochondrial oxidative capacity is in excess of the oxygen delivered to the muscles (Boushel et al., 2015). This would allow the mitochondrion to be resilient to any loss in volume or amount.

COX-IV complex catalyzes the final step in the mitochondrial electron transfer chain, and is regarded as one of the major regulation sites for oxidative phosphorylation. Dysfunctional COX-IV leads to a compromised mitochondrial membrane potential and a decreased ATP level inside the cell (Li, Park, Deng & Bai, 2006). Changes in COX-IV may therefore be an indicator of mitochondrion activity or content.

Another good marker for mitochondrial content is the content and activity of Citrate Synthase (CS) (Moberg et al., 2017). This enzyme is considered to be an important control point for the cellular metabolic rate, because it controls the first step in the TCA where it catalyzes the condensation of oxaloacetate and acetyl coenzyme A to produce citrate plus coenzyme A. In contrast to the other steps in TCA, the cell possess no alternative enzyme to CS in the event of reduced levels of CS. The synthesis of CS is suppressed by glucose and the absent of oxygen,

and elevated by the presence of oxygen and acetate (Park, McCabe, Turna & Gunsalus, 1994).

Mitochondrial activity could also be investigated by looking at enzymes that are involved in FAO, as this is an essential physiological response to energy depletion caused by fasting, severe febrile illness or increased muscular activity. Hydroxyacyl CoA dehydrogenase (HADH) is, for example, involved in the second step of FAO and catalyzes the conversion of Hydroxyacyl CoA to Keto acyl CoA. The reaction utilizes NAD^+ as a cofactor (Kapoor, Heslegrave & Hussain, 2010).

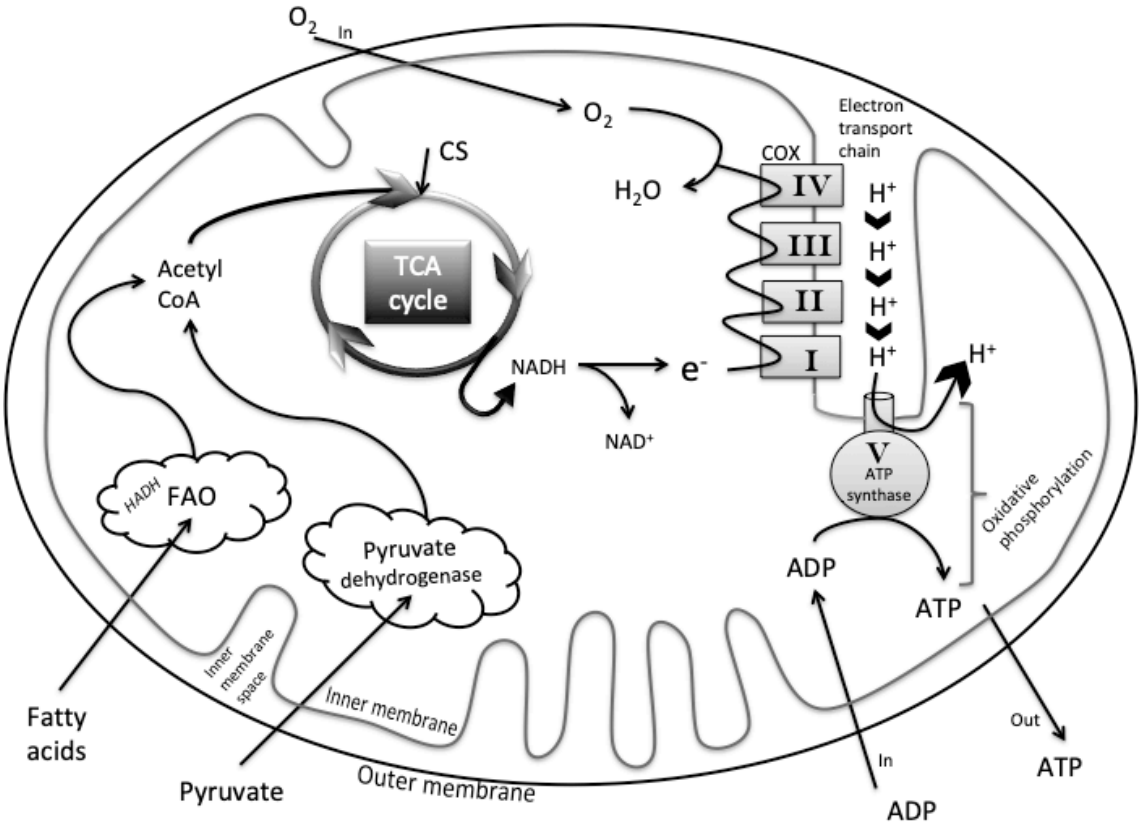


***Figure 2**: A simplified overview of the ATP regulation in the mitochondria. Modified from (Alberts et al., 2013)*

2.5 Sex differences

2.5.1 Body composition

Sex differences originate from sex specific regulation of hormones and genetic expression (Bækken & Teien, 2016; Hunter, 2016). Most of the observed differences in adults are a result of the maturation processes during puberty and adolescence, where vast changes occur for both sexes, especially in height, body mass and body composition (Epstein et al., 2013). The men have a longer and greater growth period together with a higher level of the sex hormone testosterone. The women have a higher accumulation of fat and essential fat (Bækken &

Teien, 2016). These changes are highly correlated to individual changes in hormone levels (Epstein et al., 2013).

When comparing men and women, in their twenties, some of the differences displayed are men being 13cm taller and 14-16kg heavier than women (Bækken & Teien, 2016; Epstein et al., 2013). Men possess more muscle mass, with approximately 50% more upper body LBM and 30% more lower body LBM than women (Nindl et al., 2016). Men also have a lower fat percentage than women (Epstein et al., 2013). Women thus have a generally lower body mass and also more fat reserves than men (Friedl, 1995; Pasiakos & Margolis, 2017).

2.5.2 Physical performance

On average, men are stronger compared to women, which is also true for military personnel (Bækken & Teien, 2016; Hoyt et al., 2006; Hunter, 2016). Compared to men, women generally have 40-60% less muscle strength in the upper body and 25-40% less muscle strength in the lower body (Bækken & Teien, 2016; Epstein et al., 2013). This is especially related to the fact that men possess more muscle mass compared to women, and that muscle mass is a main determinant of muscle force. Men also possess a greater proportional area of type 2 fibers, and together with more muscle mass, this leads to stronger and more powerful muscles in men than that of women (Hunter, 2014). The muscular endurance is also at a higher level for men than women, but the sex differences are smaller than what is observed in maximal strength. When measured in absolute values, the women have 40% lower anaerobic power than in men. Even if differences are still evident they may, on the other hand, be narrowed down if corrected for body mass and muscle mass (Epstein et al., 2013). When expressed relatively to body mass, the women may even have a better local muscular endurance, which is attributed to lower force production. This is, on the other hand, mostly apparent during low contraction intensity (Epstein et al., 2013). Depending on the training status, the performances in various tests do, however, overlap, so well-trained women may also perform better than men (Bækken & Teien, 2016).

2.5.3 Muscular fatigue

Activity-induced reduction in force or power generating capacity is known as muscle fatigue (Hunter, 2016). Even though men are considered to be both stronger and more powerful than women, the reduction in force and power may differ between the sexes when contractions at the same relative intensity are sustained or repeated (Hunter, 2014). For similar intensity isometric fatiguing contractions, women are usually less fatigable than men (Hunter, 2016).

This sex difference in fatigability is less clear during dynamic fatiguing tasks, however, because the number of studies is limited, and may vary depending on the type, velocity and intensity of the contractions (Hunter, 2016).

A study of intermittent voluntary static contractions of the *m. adductor pollicis* performed to exhaustion showed that women had a substantially smaller and slower decline in force-generating capacity, longer time to exhaustion and a faster recovery compared to men (Fulco et al., 1999). This was attributed to a possible slower depletion of muscle glycogen, greater capacity for oxidative phosphorylation in type 2 fibers and a greater local oxygen delivery in women, as well as a sex difference in the degree of muscle activation (Fulco et al., 1999). Women have also been found to be more fatigue resistant compared to men in other studies, including sprint, isometric contractions and maximal force. Two proposed explanations are evidence indicating that women have a greater muscle perfusion than men during exercise at the same relative intensity and that women possess slower contractile properties (Hunter, 2016). The lower absolute force reported in women together with a higher portion of type 1 fibers, higher fat oxidation and a potentially better preserved neuromuscular activity after fatiguing exercise, may result in women being more fatigue resistant (Epstein et al., 2013).

2.5.4 Metabolism

Women possess more fat than men, which allows women to have a lower resting metabolism (Frayn, 2010). Normally, women have a lower body mass as well, and will therefore spend less energy during the same activities. This leads to women being able to achieve energy balance more easily when consuming the same as men, because they are likely to have lower total energy expenditure (Friedl, 1995; Pasiakos & Margolis, 2017). The energy deficit also needs to be higher to loose weight for people of similar body mass with more initial body fat (Frayn, 2010). Women, having more body fat, may therefore loose less weight than men for the same energy deficit.

Women are further able to utilize the fat reserves in a greater manner than men at comparable exercise levels (Friedl, 1995; Pasiakos & Margolis, 2017). During physical activity, women oxidize fat at a higher rate and carbohydrate at a lower rate compared to men (Pasiakos & Margolis, 2017). One reason may be that women have more fatty acid transport proteins, which increases the availability of fat as fuel (Nindl et al., 2016). Women are also suggested to have a higher capacity for beta oxidation and increased enzymes related to lipid oxidation compared to men, whereas the opposite is the case for glycolysis, glycogenolysis and

glycolytic flux (Nindl et al., 2016). Minimalizing the use of glycogen during periods of reduced caloric intake would decrease the protein used for gluconeogenesis and may reduce losses of LBM (Hoyt et al., 2006). However, in the recovery phase after physical activity, the glucose oxidation rates are higher in women than in men whereas the men increase their fat oxidation. This has been suggested as a mechanism in women to preserve fatty acids in preparation for the next exercise bout (Nindl et al., 2016).

2.6 Effects of a strenuous field exercise

2.6.1 Content of a strenuous field exercise

Soldiers in combat may be exposed for multiple stressors, including food restriction, sleep deprivation, environmental challenges, prolonged low intensity physical work, and an element of anxiety produced by harassing opposition forces and constant performance evaluation (Shippee et al., 1994). The purpose of strenuous military training is, therefore, to prepare the soldiers to perform effectively in approaching a realistic, tactical environment under mental and physical stress found in combat, where their performance may determine life or death as the ultimate consequence (O'Hara et al., 2014; Shippee et al., 1994). The activities may vary from long periods of low- intensity to short periods of very high intensity (Margolis et al., 2014). Examples of activities may include, but is not limited to, 10km marching with 30kg loads, infiltration of an enemy position during the day and night, low crawl, wall climbing or dragging ammunition boxes while under enemy fire (Castellani, Delany, O'Brien, Hoyt, Santee & Young, 2006). The soldiers may in some situations be required to conduct physical movements for about 20h/day, which obviously may lead to extremely high daily energy expenditure (Henning, Park & Kim, 2011). The daily energy requirement during short-term FEX (< 1 week) may be as high as 6000kcal/d during, or even greater than 8000kcal/d (Hoyt et al., 2006; Opstad & Aakvaag, 1981). Even if soldiers may be compared to sporting athletes, they are, however, not always able to consume an optimal amount of energy. During these periods, the energy deficit can be as high as 40%, or more, of the total energy needs (Margolis et al., 2014).

2.6.2 Body composition and hormones

Strenuous military training may result in changes of the body composition and in large declines of body mass (table 1). Reductions in body mass is, however, widely varied, from a small decrease of only 0.1% during eight days of combat training to a substantial decrease up to 16% after eight weeks of U.S. Ranger training (USRT) (Nindl et al., 1997; Shippee et al.,

1994; Tassone & Baker, 2017). More commonly, however, seems to be a loss of 4-6% body mass during strenuous FEX with the duration of eight to twelve days (table 1) (Hamarsland et al., 2018; Margolis, Rood, Champagne, Young & Castellani, 2013; Tassone & Baker, 2017).

Generally, LBM, has been the viewed as the determining factor for physical performance in relation to military service (Grumstrup-Scott & Marriott, 1992; O'Hara et al., 2014). Losses of LBM will reduce the muscle CSA and negatively affect the force-generating capacity. The FFM losses during eight weeks of USRT has been reported to decrease approximately 6%, but 20% loss of FFM have also been reported for some individuals (Nindl et al., 1997; Shippee et al., 1994). A negative correlation has, in this regard, been observed between initial FM and loss of LBM during strenuous exercise, suggesting that a higher level of FM protects against loss of LBM (Hamarsland et al., 2018). Soldiers with less than 10% body fat are, in example, thought to be less likely to succeed the eight weeks USRT, compared to slightly fatter soldiers (Friedl, 1995). The losses of FM during this training seems to be enormous, ranging from -22.5% to - 63.4% of initial FM (Nindl et al., 1997; Shippee et al., 1994). This large variation could potentially be attributed to different needs for preservation of LBM. 64 days of U.S. Small Units Tactics training decreased the soldiers FFM with 2%, whereas the decrease in FM was as high as 26% (Margolis et al., 2013). One week of Norwegian SF training reduced the LBM by 4.5%, whereas the FM decreased 37% (Hamarsland et al., 2018). If FM preserves losses of LBM, it is likely that a reduction in LBM is reflected in a greater loss of FM.

Signals of physiological maladaptation may be sent rapidly by responding hormones, which serve as the body's chemical messengers. Insulin-like growth factor 1 (IGF-1), which promotes amino acid uptake and enhance protein synthesis, has been promoted as a biomarker reflecting physiological status, especially for physically active populations, such as military soldiers (Nindl, 2009). Long-term military operations have reported dramatic decreases in total and free IGF-1, whereas also short-term military training may result in reduced levels (Henning et al., 2014; Nindl, 2009). This decrease is also observed for total and free testosterone for long-term strenuous military training, while cortisol concentration seems to increase (Friedl, Moore, Hoyt, Marchitelli, Martinez-Lopez & Askew, 2000; Hamarsland et al., 2018; Henning et al., 2014). During energy restriction, declines in testosterone leads to preferential catabolism of type 2 fibers, that further leads to a greater proportion of muscle area covered by type 1 fibers (Russell et al., 1984; Shippee et al., 1994).

Table 1: *Overview of studies investigating the effect of FEX on body mass and body composition.*

Reference	Days	% change in BM	% change in FM	% change in LBM/FFM
Consolazio et al, 1979	10	-3.6% to -5% in ≤1500 kcal/d ↔ in 3500 kcal/d	-12.6% to -18.6% in ≤1500 kcal/d ↔ in 3500 kcal/d	-0.1% to -1.9% LBM in ≤1500 kcal/d ↔ in 3500 kcal/d
Askew et al, 1987	30	-1.6% / -5%	-12.6% / -19.7%	0.3% / -2.3% FFM
Legg & Patton, 1987	8	-1.9%	-7.1% (of % body fat)	"
Jacobs et al, 1989	5	-2.4% / -1.4%	"	"
Viswanathan et al, 1991	8	-0.1% / -0.4% / -1.2%	"	"
Shippee et al, 1994 / Johnson et al, 1994	62	- 15.6% / -12.6%	~ -50%	-6.9% / -6.1% FFM
Guezennec et al, 1994	4	↓ all groups (not reported)	"	"
Nindl et al, 1997	62	-11.3%	-42.6%	-6.6% FFM
Booth et al, 2002	23	-5.4%	↔	"
Nindl et al, 2002	3	-3.1%	-7.3%	- 2.3% LBM
Booth et al, 2003	12	-1.7% / -2.6%	-20% / -18% (of % body fat)	"
Rintamäki et al, 2005	12	-1.7%	"	"
Hoyt et al, 2006	7	-9.6% M / -9.6% W	-28.3% M / -21.5% W	-6.3% M / -5.6% W
Nindl et al, 2007	62	-12.6%	-58%	- 4.1% LBM
Welsh et al, 2008	8	-4.1%	-12.7%	-2.4% FFM
Christensen et al, 2008	8	-3.7%	"	- 5% FFM
Thorlund et al, 2011	8	- 3.3%	↔	- 4,9% FFM
Sporis et al, 2012	62	-1.4%	-19.7% (of % body fat)	"
Tanskanen et al, 2012	8	-2.1%	-16.5%	↔ FFM
Margolis et al, 2013	64	-5%	-26%	-2% FFM
Margolis et al, 2014	7	-2.5%	"	"
Henning et al, 2014	62	-8.4%	-53.6%	"
Hamarsland et al, 2018	7	-6.6%	-37%	- 4.5% LBM
Moberg et al, 2018	8	-4.6% M / -4.1% W	"	"
Raustøl, 2018	5	-8.2% M / -3.9% W	-44% M / -25.8% W	-6.2% FFM in men, ↔in women

" = not reported/measured. / = different groups. BM= Body mass. FM= Fat mass. LBM = Lean body mass. FFM= Fat-free mass. ↔ = no change. ↓= decreased. M = men. W= women.

2.6.3 **Physical performance**

Military exercises have shown detrimental effects of the soldiers' muscle force-generating capacity in various tests (table 2), both in maximal muscle force (Hamarsland et al., 2018; Nindl, Barnes, Alemany, Frykman, Shippee & Friedl, 2007; Shippee et al., 1994) and explosive muscle force (Hamarsland et al., 2018; Margolis et al., 2014; Moberg et al., 2017; Nindl et al., 2007; Nindl et al., 1997; Raustøl, 2018; Shippee et al., 1994; Welsh, Alemany, Montain, Frykman, Tuckow, Young & Nindl, 2008). The extremely demanding eight weeks USRT has shown up to 20% and 24% reduction in dynamic strength, and 21% decline in jump power (Friedl, 1995; Johnson, Friedl, Frykman & Moore, 1994; Montain & Young, 2003; Nindl et al., 2007; Nindl et al., 1997). The "hell week" of Norwegian marine soldiers resulted in a 20% reduction in maximal leg press strength and 28% reduction in jumping performance (Hamarsland et al., 2018). Declines in lower body power output have also been reported to occur after 72h or rigorous training (Nindl, Leone, Tharion, Johnson, Castellani, Patton & Montain, 2002). Even with as little as 2% reduction in body mass during seven days of winter FEX show progressively declines in both vertical jump height and lower body peak power (Margolis et al., 2014). Not all demanding FEX results in large reduction of performance, though. Results from a study on nine weeks of Croatian special operations battalion training showed a 18.9% reduction in number of pull-ups, whereas only a 6.2% decrease in maximal hip thrust (Sporis, Harasin, Bok, Matika & Vuleta, 2012). No reduction in performance and even increased physical performance has been reported as well, which was attributed to not over-utilizing the muscle groups that were tested, as well as having adequate recovery (Rintamäki et al., 2005). Immobilizing FEX, like reconnaissance missions, have, on the other side, showed as much as 8.2% and 9.9% reduction in jumping performance as well as 11% reduction in maximal voluntary isometric force, without being intentionally exposed by energy deficit (Christensen et al., 2008; Thorlund et al., 2011). The different results indicates that the design of a FEX contributes to how much the physical performance may be affected, depending on factors like duration, total energy deficit, the amount of physical activity and the tests chosen. Most observations are, nonetheless, that physical performance declines markedly after a strenuous FEX.

Table 2: *Overview of studies measuring effect of FEX on physical performance.*

References	Characteristics of the field exercise	Change in performance
Consolazio et al, 1979	10d maneuver in the Panamanian jungle with VEI	↓ VO_2max in 600/1500kcal/d, ↔ VO_2max in 1000/3500 kcal/d ↔ 15miles
Askew et al, 1987	30d U.S. SF training with VEI	↓ VO_2max, ↑ Isometric HS, ↓ Army physical test, except ↑10km in 1950kcal/d ↓ Isokinetic strength and ME in 1950kcal/d and ↑ in 3600kcal/d
Legg & Patton, 1987	8d of combat artillery field training.	↓ UB power, ↓ Isometric HS, ↑ Wingate
Jacobs et al, 1989	5d FEX for Canadian Forces with VEI	↓Isokinetic strength, ↓peak VO_2, ↓ Wingate test, ↓UB ME
Viswanathan et al, 1991	8d patrol operation with VEI	↑ VO_2max, ↑ Modified Harvard Step Test ↔ Hill climb, ↔ OC
Shippee et al, 1994 / Johnson et al, 1994	62d of USRT with VEI.	↓ MVC LB ↓ JH, ↓ Jump power ↔ HS and handgrip endurance in 2820kcal/d
Guezennec et al, 1994	5d mountain special operation with VEI	↓ VO_2max in 1800kcal/d and ↔ in ≥ 3200kcal/d, ↔ Anaerobic performance
Nindl et al, 1997	62d of USRT	↓ MVC LB, ↓ JH, ↓ Jump power
Booth et al, 2002	23d of tropic military training	↓ Jump power, ↔ ME , ↑ HS, ↑ beep test
Nindl et al, 2002	3d U.S. sustained operation	↓ WB, ↓ LB power, ↓ ME, ↓OC, ↔MVC, ↔UB power, ↔GT, ↔Marksmanship
Booth et al, 2003	12d tropical training with VEI	↑ HS, ↑ 2.4km run, ↔ UB strength
Rintamäki et al, 2005	12d winter training	↑/↔JH, ↔Isometric MVC LB, ↔VO_2max, ↔Jump power
Nindl et al, 2007	62d USRT	↓ MVC LB, ↓ JH, ↓ Jump power
Welsh et al, 2008	8d U.S. sustained operation	↓ LB mean power ↓ JH for 1 and 5 repetitions, ↔ JH for 30 repetitions
Christensen et al, 2008	8d immobilizing mission	↓ Isometric MVC, ↓Contractile RFD, ↓CMJ
Thorlund et al, 2011	8d immobilizing mission	↓ Isometric MVC, ↓Contractile RFD, ↓CMJ
Sporis et al, 2012	62d special operation battalion training	↓MTB, ↓ME, ↓ MVC LB ↓Sprint, ↓3.2km, ↔ JH, ↔ Agility
Tanskanen et al, 2012	8d Finnish winter training with VEI	↔ JH, ↔ HS, ↑ 3km, ↑ Jump power
Margolis et al, 2014	7d Norwegian winter training	↓ JH ↓ Jump power
Hamarsland et al, 2018	7d selection course for Norwegian Naval SF	↓ CMJ, ↓ MVC UP and LB
Moberg et al, 2018	8d Swedish military training	↓ Jump height, ↓ HS
Raustøl et al, 2018	5d selection course for Norwegian SF	↓ CMJ, ↓ power, ↓Anaerobic work capacity, ↓ MTB

↓= decrease. ↓= increase. ↔ = no change. VEI= Varying energy intake. d= Days. SF= Special Forces. USRT= U.S. Ranger training. LB = Lower body. UB = Upper body. MBT=Medicine ball throw. RFD= Rate of force development. VO_2max= Maximal oxygen uptake. HS= Handgrip strength. MVC= Maximal voluntary contraction. ME= Muscular endurance. CMJ= Countermovement jump. JH= Jump height. GT= Grenade Throw. WB= Wall building. OC= obstacle course

2.6.4 The role of body mass on physical performance

The nature of military operations makes it challenging to achieve energy balance. High amount of physical activity causes great energy expenditure, whereas previous operational experiences recognize that soldiers often endure periods of limited food and consequently low energy intake (Montain & Young, 2003). A study manipulating the soldiers' energy intake during ten days FEX illustrated that only the soldiers having matched energy intake and energy expenditure avoided reductions in body mass, whereas all the other groups experienced reductions (Consolazio, Johnson, Nelson, Dowdy & Krzywicki, 1979). Underfeeding, and thereby negative energy balance, consistently demonstrates detrimental effects on physical performance, if conducted long enough (Montain & Young, 2003). Military leaders have recognized this problem and several studies have been made to enhance field-feeding (Pasiakos & Margolis, 2017). A systematic review by Tassone & Baker (2017) on the use of combat rations in military training or deployment argues that the body mass loss needs to exceed 5% of initial body mass to have any detrimental effect on performance, and that this is likely to occur during FEX with durations of eight to twelve days (Tassone & Baker, 2017). For FEX of shorter duration they argue that even if the energy deficit is high, it will not influence the performance, because body mass losses are < 5% (Tassone & Baker, 2017). A winter FEX over twelve days showed a small body mass loss of only 2%, which was not sufficient to cause any detrimental effects on physical performance (Rintamäki et al., 2005). We do not know, however, how the energy balance was for the soldiers in this study. During the energy manipulating study, similar body mass losses of < 5% was displayed independent of energy deficits of approx. 3000, 2600 or 2100/d (Consolazio et al., 1979). This could indicate a mechanism to preserve the body mass for short-term periods, even under severe energy deficits. It is, nonetheless, suggested that short-term periods of underfeeding have limited impact on physical performance, and that it most likely is the magnitude of energy deficit and number of days being underfed that eventually compromises it (Montain & Young, 2003). An energy deficit of 900/day during USRT over eight weeks resulted, in example, in 13% loss of body mass (Shippee et al., 1994). This illustrates that when maintained for a longer period, even a smaller energy deficit may cause severe reductions in body mass. The US Institute of Medicine Committee of Military Nutrition Research states that <10% loss

of body mass is unlikely to affect performance. They do, on the other hand, also state that >10% loss of body mass is very likely to impact physical performance (Institute of Medicine Committee on Military Nutrition, 1995). It also seems to be a general agreement amongst researchers that a reduction in body mass of more than 10% has huge consequences for a soldier's performance (Friedl, 1995; Henning et al., 2011; O'Hara et al., 2014; Shippee et al., 1994). As discussed in earlier sections, LBM is important for physical performance and this statement is therefore probably related to that large LBM decrements primarily occur when there are substantial losses in body mass and when fat mass gets close to critical low values. Because the loss of body mass is reflected by substantial losses is FM, having large initial values of FM could contribute to prevent LBM losses. There are, however, studies showing decrements in physical performance even with < 5% loss of body mass (Margolis et al., 2014). Furthermore, a review study shows that the correlations between body composition and maximal lifting performances only range from being poor- to-fair predictors of maximal lifting performances (Hydren et al., 2017). It is therefore not necessarily detected reliable correlations between loss of body mass and physical performance. The relative decrements in physical performance reported during USRT were, in example, greater than body mass and fat-free mass (Nindl et al., 1997). The recovery of body mass has further been reported to occur faster than the recovery of physical performance, which mitigates energy deficits and loss of body mass as explanation for the reduced physical performance (Hamarsland et al., 2018). The measurement of handgrip strength has been a much used test to assess the impact of dietary restriction on muscle strength, but only one of eight studies using this test has documented a loss in muscle strength over a range of 2-16% body mass loss (Montain & Young, 2003). The relationship between body mass and physical performance is therefore a truth with modifications, especially when the body composition isn't considered.

2.6.5 **Sex differences**

Most of the existing studies on FEX have been based on men solely, and so the information on how the women respond to a strenuous FEX is lacking. In example, a recent review of 30 military studies on field rations, investigated only one study that included two women. Nonetheless, this review suggest that women may tolerate a lower body mass or body composition changes before any health and/or performance decrements occur, due to the larger fat reserves (Tassone & Baker, 2017). There are to

the author's knowledge, however, four studies investigating the sex differences in response of a strenuous FEX (Castellani et al., 2006; Hoyt et al., 2006; Moberg et al., 2017; Raustøl, 2018). One of these studies sought to determine whether performing the same work tasks during a 54h strenuous FEX would result in equal energy expenditure for men and women. They concluded that women did expend less energy than men, but that men and women showed no difference in total energy expenditure when expressed relative to body mass or fat-free mass (Castellani et al., 2006). The same findings have been reported by later studies (Hoyt et al., 2006; Raustøl, 2018), but not all (Moberg et al., 2017).

The decline in body mass after the 54h FEX was significantly different between the sexes with men losing 3.1% body mass, whereas women only lost 1.6% (Castellani et al., 2006). A study conducted on Norwegian cadets during 7 days FEX reported also a lower decreased in body mass for women (-5.95kg) compared to men (-7.47kg) (Hoyt et al., 2006). Reductions for both sexes were observed also in a recent study of sex differences in SF conscripts during a five-days FEX, with women decreasing 2.67% and men decreasing 6.5% (Raustøl, 2018). Moberg (2017), however, found no significant differences in loss of body mass between the sexes after a Swedish 8 days strenuous FEX, even though women showed a slightly smaller reduction of 4.1%, whereas men lost 4.6% (Moberg et al., 2017). The results seem to be consistent in that both sexes loose body mass, and that women declines less compared to men. The body mass losses seem to increase for both sexes with longer duration of a FEX.

Three studies report on sex differences in body composition after a strenuous FEX. After the 54h FEX, there were found no differences between sexes in neither FM nor LBM. During the five days FEX, the men reduced their FFM with 2.67%, while women displayed no change (Raustøl, 2018). Hoyt (2006) found sex differences in absolute FFM where men lost 4kg, whereas women only lost 2.5kg. The percentage loss of FFM, however, did not differ between the sexes (Hoyt et al., 2006). Both sexes had losses in FM in the five-days FEX, but there was a larger absolute reduction in FM in women (2.79kg) compared to men (1.84kg) (Raustøl, 2018). For FM, Hoyt (2006) report that the absolute losses of FM did not differ between the sexes, but that the percentage loss of FM was significantly higher in men (28.3%) than in women (22.1%) (Hoyt et al., 2006). It is therefore not conclusive how the body composition changes for the sexes

after a FEX, but there are indications that men loose more LBM than women. The reason for different changes in body composition for the sexes after a strenuous FEX may be caused by a different substrate oxidation, which is only reported by Hoyt (2006). In that study women had higher fat oxidation rater per kg muscle FFM than men (Hoyt et al., 2006).

Differences in physical performance have been reported by two recent studies (Moberg et al., 2017; Raustøl, 2018). The five-day FEX showed similar decrease for both sexes in maximal power, anaerobic work capacity, medicine ball throw and countermovement jump (CMJ) performance after the FEX (Raustøl, 2018). Reductions found in CMJ or handgrip strength was also not different between the sexes in the Swedish eight days FEX (Moberg et al., 2017). This indicates that a strenuous FEX causes equal declines in performance for both sexes.

2.6.6 **Myocellular response**

Exercise stimulates repeated increases in mRNA expression, which results in enhanced translation of proteins and ultimately adaptive changes in muscle protein content (McGlory et al., 2017). A military operation normally consists of periods with low- and high-intensity exercise, but it also normally contain insufficient food intake. Both exercise and nutritional intake regulates the rates of skeletal muscle protein synthesis and/or muscle protein breakdown, which determines the total skeletal muscle protein balance (Moberg et al., 2017). There are few studies investigating the myocellular response of a strenuous FEX, and only Moberg (2017) has investigated if there are any sex differences in muscle glycogen, metabolic proteins, protein content and phosphorylation and proteasome activity after a strenuous FEX. No differences were found between the sexes (Moberg et al., 2017).

Measurement of protein balance in the Swedish eight days strenuous FEX showed that a physically demanding military operation could lead to an induction of autophagy (Moberg et al., 2017). This study found increased AMPK-mediated phosphorylation of Ulk1, together with increased total protein levels of Ulk1. It was therefore suggested that a prolonged and exhaustive exercise period induces an increased capacity to stimulate autophagy. The increased activity and total protein levels of Ulk1 further implies an increased activation of AMPK, which was also supported during this exercise by an observation of increased phosphorylation of $AMPK^{Thr172}$ (Moberg et al.,

2017). However, in other studies with energy deficit, fasting, low-intensity exercise or a combination of the latter two, there has been reported no change in the phosphorylation of AMPK^{Thr172} (Areta et al., 2014; Moberg et al., 2017; Schwalm et al., 2015).

Endurance and resistance training in combination may cause attenuated adaptations to muscle hypertrophy (Fyfe et al., 2018). Both endurance and strength capacities are challenged during a military exercise and the energy balance is also mostly negative during a FEX. It can therefore be expected no change, or perhaps a reduction, in activation of proteins involved in the regulation protein synthesis during FEX despite high levels of physical activity. These expectations were met during the Swedish 8-days strenuous FEX, with evidence showing no change in total protein levels in S6K1, mTORC1, 4E-BP1, or eEF2, and also no change in 4E-BP1^{Thr46} or eEF2^{Thr56} (Moberg et al., 2017). It can therefore be argued that proteins involved in the regulation of protein synthesis are not affected by a military FEX including nutrient deprivation. Another seven days FEX did, on the other hand, show an increase in protein synthesis (Margolis et al., 2014). This increase may be related to synthesis of heat-shock proteins, which has been observed after damaging eccentric exercise. Increased levels of heat-shock proteins may occur to protect cytoskeletal and myofibrillar structures during exercise, and may also increase protection against future situations of oxidative and mechanical stress (Paulsen et al., 2009). The seven days FEX also showed an increase in protein breakdown which was greater than the increased protein synthesis (Margolis et al., 2014). It can therefore be expected that the protein balance is negative after a strenuous FEX. One interesting finding, from three days of arctic military training, was that even if protein synthesis declined, the protein breakdown was also reduced, resulting in a down regulation of the whole-body protein balance, which was suggested to occur as an adaptive response to spare protein (Margolis et al., 2016).

Energy deficit is one determining factor contributing to reduce mTORC1 signaling (Moberg et al., 2017). In reconnaissance missions, almost 24h/day is conducted in prone position and with little to no movement. The soldiers are still exposed to sleep deprivation and energy deficit, but physical activity is almost not present. A study conducted on a eight days FEX designed like this, showed both increase in protein breakdown and decrease in protein synthesis (Jespersen et al., 2015). The protein levels of FoxO3, atrogin1 and MuRF1 were elevated, indicating an activation of protein

breakdown. There was also a decrease in mTOR content, while other protein synthesis markers remained unchanged, which indicates a reduced protein synthesis rate (Jespersen et al., 2015). The overall protein balance seems therefore to be negative, even if the physical activity level was low. These findings suggest that physical activity, even if it may cause a greater energy deficit, may be an important stimulus to keep the protein synthesis at a desired state when exposed to energy deficit. It also emphasize the impact restricted caloric intake alone has during a FEX.

Strenuous FEX does not seem to alter the activity of HAD or CS, which argues that these kinds of exercises does not have significant impacts on the mitochondrial content or activity (Moberg et al., 2017). A four weeks demanding ski journey across Greenland, resulting in a large decrease in body mass, also showed unchanged CS (Helge et al., 2006). Both of these findings support the suggestion that the muscle mitochondria are working at submaximal flux rate under normal conditions, and are therefore able to work sufficiently during less favorable conditions (Vikmoen, 2015). There may be region specific variations, on the other hand. A study on five weeks skiing showed, for example, increased oxidative enzymes and capillary density in *m. triceps brachii*, with no changes in *m. deltoideus* or *m. vastus lateralis* (Schantz, Henriksson & Jansson, 1983). They did not, however, report on the changes in body mass during the study. In a study of 42 days skiing, there was, on the other hand, a 23% reduction in the mitochondrial oxidative phosphorylation capacity in the *m. quadriceps*, indicating reduced mitochondrial content and which was also supported by a reduction in CS (Boushel et al., 2015). There was, however, no change in HAD. The reduction in mitochondrial content and volume in that study was suggested to happen because of activated mitophagic pathways, which was though to be activated as a mechanism to achieve bioenergetic efficiency. The reduction in mitochondrial oxidative capacity did not, however, affect the maximal oxygen uptake (Boushel et al., 2015).

2.7 Recovery period after FEX

The time course of recovery to normal physical function of a soldier is of great importance to military applications, as it allows for appropriate planning of recovery periods between repeated bouts of strenuous military training or deployments of combat units (Shippee et al., 1994). Prolonged exhaustive military activities may lead to prolonged reductions in physical capabilities and cognitive functions, but may also

affect the ability to recover from any possible infections or injuries (Friedl, Mays, Kramer & Shippee, 2001).

There are still only few studies that have investigated the recovery time of physical capabilities in the aftermath of a strenuous FEX, including both women and men. To the author's knowledge, there are no previous studies investigating the recovery of myocellular alterations of a strenuous FEX.

2.7.1 Body composition and hormones

Because body mass and composition have been considered important in estimating the physical ability of a soldier (Tassone & Baker, 2017), it is of interest to understand how the recovery period elapses and if the different components of body composition recover differently. One of the earliest studies investigating recovery after strenuous military training was conducted on eight weeks of USRT. This study showed full recovery of both body mass and body composition after five weeks of rest (Nindl et al., 1997). Actually, the body mass and FM had increased above pre-values with 7.1% and 62%, respectively. Accelerated fat storage in adipose tissue may be a result during weight regain after caloric restriction, because of suppressed skeletal thermogenesis (De Andrade et al., 2015). Recovery of body mass and fat percentage was also reported two to six weeks after another Ranger Course, where there were no significant increased in either body mass or percentage fat (Henning et al., 2014). Considering these two studies, it is indicated that body mass and body composition are recovered at least within five weeks, and that the recovery may occur at an earlier stage. Hamarsland (2018) investigated a recovery process two weeks after strenuous FEX. This study showed that the recovery time of body mass after a "hell week", for male soldiers in the Naval SF, was only one week. Muscle mass was also restored to normal after one week of rest, whereas fat mass was still reduced after one week of rest (Hamarsland et al., 2018). During the five days FEX for the SF, the body mass and FFM was recovered after 72h of rest, while FM needed one week of rest (Raustøl, 2018). A study investigating the effect of a 32 days crossing of Greenland by ski, showed that the body mass, FM and LBM had not recovered to baseline for the subjects after three to four days rest, with the decline in body mass consisting primarily of losses in FM (Helge et al., 2006). These studies indicate that body mass and composition is likely to recover within one week of rest, following strenuous FEX. We do not know, however, if energy deficits during longer deployments (> 8 weeks) follow the same pattern.

It has been reported that, during the recovery period after a strenuous FEX, reduced serum hormone levels of testosterone and IGF-1 recover within five weeks, but that they may also recover fully after only one week of rest (Friedl et al., 2000; Hamarsland et al., 2018). An increased cortisol level, on the other hand, seems to need longer than one week to recover to normal levels (Friedl et al., 2000; Hamarsland et al., 2018). The recovery of endocrine changes may be dependent on the status of glycogen, and may explain why some studies show a faster recovery than others (Hamarsland et al., 2018; Henning et al., 2011). One study of a USRT investigated the recovery of endocrine and cytokine measurements, but they did not report when the different participants were tested, and therefore concluded that the endocrine and inflammatory status returned to homeostasis within two to six weeks of rest (Henning et al., 2014). A report on all studies conducted on the Norwegian Army War Academy concludes that changes in hormones after a strenuous FEX recovers within 23 days (Teien, 2013). The endocrine and immune response on a FEX, like reduced testosterone/cortisol level, decreased iron status, decline in immune function and decreased IGF-1, are also seen in overtraining. An interesting aspect in this regard, is that studies on recovery from overtraining have been reporting recovery times over weeks, months or even years (Cadegiani & Kater, 2017; Peake, Neubauer, Walsh & Simpson, 2017; Tanskanen et al., 2011). A big difference between a strenuous FEX and overtraining in elite athletes is notably that overtraining is not necessarily linked to sleep or food deprivation. Even so, the interactions between immune and hormone levels and effects on muscle tissue are complex, and are not directly transferable conclusions. These measurements are therefore not necessarily sensitive enough to explain the recovery of physical performance.

Table 3: *Overview of studies investigating the recovery of body mass and body composition after a FEX.*

Reference	RTI	TTR body mass	TTR FM	TTR LBM/FFM
Consolazio et al, 1979	8 days	NR ≤1500kcal/d 7 days in 3500kcal/d	7 days	NR
Nindl et al, 1997	5 weeks	5 weeks	5 weeks	5 weeks
Henning et al, 2014	2-6 weeks	2-6 weeks	2-6 weeks	"
Hamarsland et al, 2018	2 weeks	72h	NR (1 week)	1 week
Raustøl, 2018	2 weeks	72h	1 week	0h W / 72h M

RTI= Recovery time investigated. TTR = Time to recover. NR = Not recovered within RTI. " = Not reported/measured. / = Different groups. FM= Fat mass. LBM = Lean body mass. FFM= Fat-free mass. ↔ = no change. ↓= decreased. M = men. W= women

2.7.2 **Physical performance**

Investigation of the acute recovery of a strenuous FEX show that three hours rest can result in a slight recovery in some of the reduced muscle force-generating capacities. After 8 days of an immobilizing FEX, MVC force of the knee extensors and jump height showed slight recovery after three hours of light activity and nourishment, but remained decreased by 8.9% and 6.2%, respectively. There was also present a 20% decline in RFD, which did not show any recovery after three hours (Thorlund et al., 2011). Another similar FEX displayed an even greater recovery of jump height after three hours of rest, from 8% reduction up to only 2.9% reduction. The soldiers did, on the other hand, experience a 10% and 21% reduction for the knee extensors strength in MVC and peak RFD, respectively, which did not recover after three hours (Christensen et al., 2008). A rapid initial recovery of squat jumps after 90 minutes of high-intensity eccentric exercise was observed during the first eleven hours and was followed by a second drop in performance 22h after exercise (Raastad & Hallén, 2000). A second drop in jump performance and maximal leg power was also observed for men in the recovery phase between 24h and 72h after a selection exercise for the Armed Forces SF (Raustøl, 2018). For the Naval SF the same drop was present between 24h and 72h in maximal leg press, but not for CMJ (Hamarsland et al., 2018). It is possible that the mechanism seen in eccentric training occurs in the recovery phase of strenuous military training, and also for the initial recovery seen in immobilizing FEX.

A full recovery of isometric leg press force for Norwegian Naval SF after their "hell week" occurred after two weeks of restitution. However, their jumping capacity was still 14% reduced at this time point (Hamarsland et al., 2018). Consistent findings were found in another study of Norwegian SF, where two weeks of rest was not enough to recover the soldiers jumping capacity (Raustøl, 2018). This indicates that the physical performance may be severely impaired for several weeks after a strenuous FEX, which undoubtedly will affect the soldiers' military performance. Even short periods of strenuous exercise, without intentions of energy deficit, could display long recovery time in muscle force-generating capacities. A reduction of 13% in CMJ after a marathon race was, in example, still depressed 12% after five days of restitution (Petersen, Hansen, Aagaard & Madsen, 2007). Just like the design of an everyday training session may cause decrease in physical performance, the design of a FEX may also explain differences seen in the recovery phase following a FEX. The total load and stress

exposed to the soldiers, together with the total amount of energy deficiency and how long the FEX is, may all be factors contributing to these differences. It is therefore difficult to estimate the exact amount of time needed to recover from a strenuous FEX. We may perhaps, based on results from a recovery study done after eight weeks of extremely demanding USRT, conclude that five weeks is sufficient to recover the explosive, as well as maximal strength (Nindl et al., 1997).

***Table 4**: Overview of studies investigating the recovery of physical performance after FEX.*

References	RTI	VO_2max	MVC LB	MVC UB	JP	Power	RFD	MTB	AW
Consolazio et al, 1979	8 d	NR ≤ 600kcal/d 5 d ≥ 1000kcal/d	"	"	"	"	"	"	"
Nindl et al, 1997	5 w	"	5 w	"	5 w	5 w	"	"	"
Christensen et al, 2008	3 h	"	NR	"	NR	"	NR	"	"
Thorlund et al, 2011	3 h	"	NR	"	NR	"	NR	"	"
Hamarsland et al, 2018	2 w	"	2 w	1 w	NR	"	"	"	"
Raustøl, 2018	2 w	"	"	"	NR	NR	"	1 w	2 w

RTI = Recovery time investigated. LB= Lower body. UB = Upper body. " = Not reported/measured. d= Days. w = Weeks. h = hours. VO_2max: Maximal oxygen uptake. MVC = Maximal voluntary contraction. JP= Jump performance. RFD= Rate of force development. MTB= Medicine ball throw. AW= Anaerobic work.

2.7.3 Sex differences

It is not yet clear whether the recovery of physical performance elapses differently for men and women after strenuous military training. A faster restitution of physical function for women may, perhaps, be connected to the previously discussed higher fat oxidation and better preservation of muscle mass (Pasiakos & Margolis, 2017). A study comparing male and female SF conscripts showed, on the other hand, similar restoration for both sexes of body mass and LBM after 72 hours of restitution, and that fat mass recovered after one week of rest (Raustøl, 2018). Neither a bout of eccentric exercise, that caused approximately 50% reduction in force-generating capacities and, which did not recover after three weeks of rest, reported any sex differences (Paulsen et al., 2009). It should, nonetheless, be noted that female SF conscripts did not decline in FFM after the strenuous FEX, whereas the men did (Raustøl, 2018). A difference observed between sexes in the recovery phase of exercise is substrate oxidation. Opposed to what

is seen during exercise, women seem to have a higher carbohydrate metabolism than men in the recovery phase. This increase in carbohydrate metabolism is suggested as a mechanism to preserve the fatty acids in order to rebuild the muscle triglyceride (Bækken & Teien, 2016; Nindl et al., 2016).

The men may potentially be more affected by a strenuous FEX than women, when there are greater losses of LBM, because they possess more muscle mass and also greater amount of type 2 fibers (Hunter, 2016; Simoneau & Bouchard, 1989). This could perhaps explain the already mentioned second drop in performance 72h after strenuous military training during the recovery phase for men, and not women (Raustøl, 2018). It has recently been presented evidence of a possible difference in the recovery of strength parameters (Raustøl, 2018). Women demonstrated, in example, a faster restoration compared to men in jumping performance in CMJ after a strenuous five days selection FEX. Neither sexes were fully recovered after two weeks of restitution, but there was a significant difference between the sexes with men showing a greater reduction in CMJ. The men also showed a slower recovery of their maximal power, and was the only sex still having significant reduction after two weeks of restitution (Raustøl, 2018). This may therefore indicate that women recover faster than men in muscle force-generating capacity. One explanation could be that women possess a larger amount of type 1 fibers, which may be more fatigue resistant (Simoneau & Bouchard, 1989). For both sexes, on the other hand, medicine ball throw recovered after one week of restitution and anaerobic work capacity after two weeks of restitution (Raustøl, 2018). We may, in any case, conclude that a full recovery of physical strength performance after a strenuous FEX is long lasting for both sexes.

It is plausible to believe that women and men have different myocellular recovery to a strenuous FEX, because of the sex specific regulation of hormones and genetic expression (Bækken & Teien, 2016; Hunter, 2016). Serum creatine kinase (CK) activity, measured by blood samples and indicator of muscle damage, has, in example, been reported to be generally lower in women compared to men in the recovery phase of a bout of eccentric training (Stupka, Tarnopolsky, Yardley & Phillips, 2001). However, we have no reports of this being different between the sexes after a FEX, and muscle damage could potentially activate both protein breakdown and protein synthesis in order to remove and replace damaged proteins. On the other hand, we might see a

greater rise in protein synthesis during the recovery phase in men than in women. If men loose more LBM than women, they would have a greater need to restore lost muscle mass. Because there are no studies investigating sex differences in myocellular recovery after strenuous military training, we are unable to predict if there are any sex differences in the myocellular response of a strenuous FEX.

2.7.4 Myocellular response

There exist no studies on cellular investigation in the recovery phase of a strenuous FEX, perhaps because muscle biopsy is an invasive technique, and we may therefore only make assumptions on how the protein balance act. However, there are studies using other measurements in the recovery phase of a FEX and we may also look to studies investigating other damaging exercises, which may give us some indications and strengthen our assumptions. A change in endocrine levels has, in example, been suggested as an explanation to changes in physical performance and recovery (Hamarsland et al., 2018). Decreased testosterone and IGF-1 indicates a reduced rate of protein synthesis, which may come as an adaptation to maintain a normal concentration of glucose in the blood. Increased cortisol indicates catabolism and probably reflects lipolytic and proteolytic effects in order to provide substrates for hepatic gluconeogenesis (Hamarsland et al., 2018; Henning et al., 2011).

A rise in protein synthesis after a FEX should perhaps not be a surprise. It has been well established that a single bout of exercise, even when performed in the fasted state, increases human skeletal muscle protein synthesis. The synthesis seems even to be stimulated to a greater extent than breakdown, and the increase has been revealed to last for several hours, and even days (Drummond, Dreyer, Fry, Glynn & Rasmussen, 2009). To restore the body mass after a FEX, and LBM in particular, would probably demand longer sustainment of increased protein synthesis than what is seen after a bout of exercise. In the recovery phase after eccentric training leading to long lasting reduced force generating capacity, it has also been observed increased synthesis of specific heat-shock proteins for up to seven days, which is probably related to muscular adaptations through protection against future situations of oxidative and mechanical stress (Paulsen et al., 2009). Protein breakdown is also stimulated by exercise and energy deficit, and an increase is therefore to be expected after a strenuous FEX (Moberg et al., 2017). Without replenishing with nutrients, the net balance of muscle proteins will remain negative (Drummond et al., 2009). Nutrition is available in the recovery phase of a

strenuous FEX and the protein breakdown rate should therefore be expected to decline, or at least remain unchanged. An activation of protein breakdown in the recovery phase could, nonetheless, be a mechanism to eliminate damaged and/or aberrantly folded proteins generated from the FEX and it could also be required for improving insulin sensitivity and mitochondrial biogenesis (Schwalm et al., 2015). Protein synthesis has, however, the decisive role of myocellular protein accumulation (Ferrier, 2014).

2.8 Possible mechanisms behind long-lasting reduction of physical performance

Of the few studies investigating physical performance during the recovery period of a strenuous FEX, it is apparent that we still do not know what causes the long lasting reduction of muscle force-generating capacity. Changes in body composition has already been discussed as a possible factor for reduced physical performance, but does not explain the long-lasting fatigue, as discussed in section 2.6.4. The use of tissue components of the body to monitor malnutrition and/or recovery was already questioned in the first recovery study after eight weeks of USRT. They suggested that this should be exercised cautiously, because components of body mass appeared to recover more rapidly than physical performance measures (Shippee et al., 1994).

Instead of atrophy being the mechanism behind reduced muscle fore-generating capacity, it is suggested that it is due to damage to the contractile apparatus within the muscle fibers. This has also been suggested as a potential mechanism behind long-lasting recovery after eccentric exercises (Hamarsland et al., 2018). The impaired myofibrillar function may occur because of disturbance, weakening or damage in one of the many parts contributing to force production, like damages to the z-discs or sarcomeres in the contractile apparatus. This seems, nonetheless, to be of more importance in the early stages of fatigue. In the later stages, the role of Ca^{2+} homeostasis, and in particular a decreased Ca^{2+} release from the SR, may be of more importance (Cheng, Place & Westerblad, 2018). Inhibiting SR Ca^{2+}-release into cytosol could reduce the amount of ATP-consuming cross-bridge cycles and also reduce the ATP-dependent re-uptake of Ca^{2+} through SERCA pumps, which would reduce the energy consumption. A correlation between reduced SR Ca^{2+}-release and reduced muscle glycogen storages has been observed (Cheng et al., 2018).

There is a lack of studies on the cellular Ca^{2+}-handling in relation to military training,

but there are other studies describing the potential role in long-lasting reduced muscle force-generating capacity. With high intensity training it has been reported that the RyR type 1 (RyR1) becomes fragmented and cause changes in Ca^{2+}-handling, involving calpain activation, increased SR Ca^{2+} leak at rest, and depressed force production because of impaired SR Ca^{2+}-release upon stimulation (Place et al., 2015). Reduced re-uptake of Ca^{2+} to the SR is therefore one proposed mechanism, because it can increase the maintained cytosolic Ca^{2+} concentrations at rest, and this can further damage proteins involved in the excitation-contraction coupling (ECC) (Place et al., 2015). It is also been proposed that increases in intracellular Ca^{2+} can stimulate AMPK phosphorylation and subsequently autophagy (Moberg et al., 2017).

A mechanism to avoid large concentrations of cytosolic Ca^{2+} is creating vacuoles in the t-tubuli system, which has the potential to store Ca^{2+}. These can be created as a response to hard exercise and may help reduce or limit the Ca^{2+}-dependent damage. Vacuoles may on the other hand interrupt the action potentials running through the longitudinal axes in the t-tubuli system, and may therefore be a reason for the force reduction seen in the days after heavy resistance training (Cully et al., 2017).

Changes in $Na^{+}K^{+}$-pump may alter the resting potential in the cell, which further alters creation of action potentials with Ca^{2+}-influx. This has been addressed as an important factor for muscle fatigue, and may also explain why the Ca^{2+}-handling do not function properly (Cheng et al., 2018). However, it cannot be excluded that a form of central fatigue or pain can be a contributing factor to reduced muscle performance, as observed after other long-lasting exercise forms (Hamarsland et al., 2018).

Measuring the expression and location of heat shock proteins may increase our understanding on how the Ca^{2+}-handling is affected, by locating where there is damage in the muscle cell (Paulsen et al., 2009). Impairments in the Ca^{2+}-handling would further be visible in tests of muscle force-generating capacities, and especially in explosive strength measurements. Because impairment in Ca^{2+}-release from SR might occur because of, and is likely to cause further, damage to proteins involved in the ECC, this could be investigated by measuring the expression of proteins in the ECC like the RyR1or t-tubular voltage sensor (Place et al., 2015) and SERCA pumps or calpains.

3. Method and materials

3.1 Participants

The participants (n=18) were recruited from individuals studying at the Norwegian Defense Cyber Academy (FIH) who were participating in a strenuous FEX in the period May-June 2018. To be able to study at FIH, the soldiers have to go through a selection process at the Armed Forces Admission and Selection (Forsvarets Opptak og Seleksjon) where the applicants must pass physical- and medical tests and interviews. During the education, the soldiers are continuously evaluated and are faced with strict criteria, which can be both physically and mentally demanding. One of the evaluations is an obligatory and challenging FEX called "Cyber Endurance".

The recruitment of participants was administered through an oral presentation of the study at the 24th April 2019 for all the soldiers that were going to participate in the FEX. The soldiers were informed about the purpose of the study and how it would be conducted, as well as any extra strains that could occur in accordance with participation and the could ask questions to the researchers. It was clarified that participation in the study was voluntary and that candidates could withdraw at any time.

Soldiers who volunteered to participate signed a written informed consent before testing commenced. A total of 10 men and 8 women volunteered to participate in the study, and their characteristics are presented in table 5. The men had significantly higher height, weight and LBM.

Table 5: Participant anthropometry prior to the FEX.

	Male (n=10)	**Female (n=8)**
Age (years)	20.5 ± 0.5	21.4 ± 1.4
Height (cm)	181 ± 6.7	165.9 ± 3.1*
Weight (kg)	76.7 ± 6.4	65.4 ± 12.3 *
LBM (kg)	38.2 ± 3.4	28.8 ± 3.7 *
FM (kg)	9.99 ± 2.58	14.11 ± 6.45 #

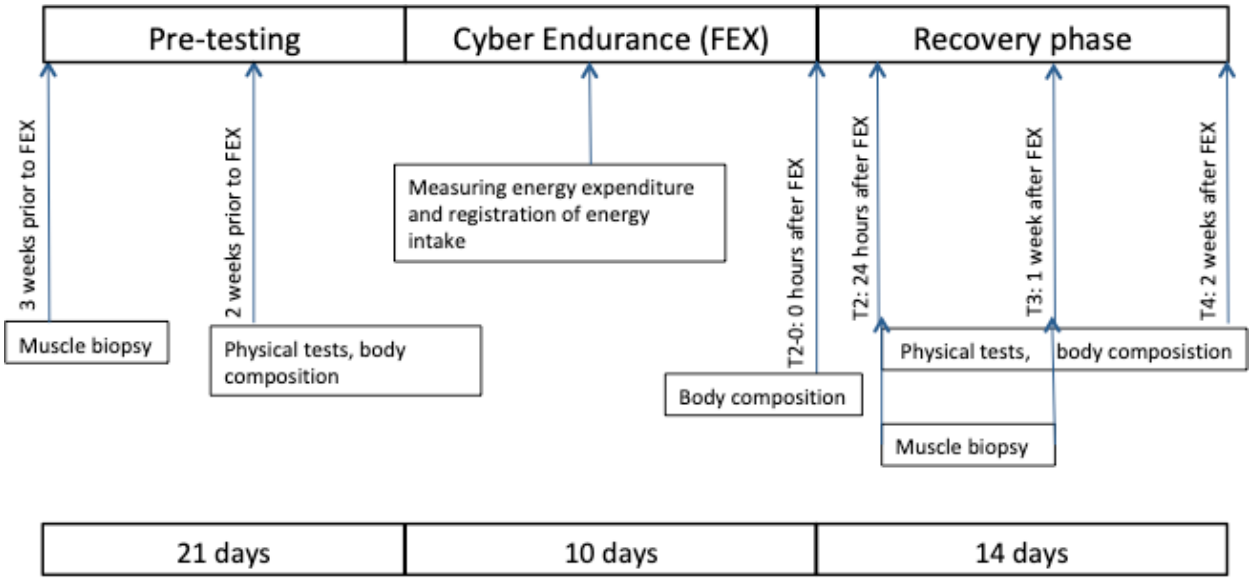
*Values are mean ± standard deviation. * = Significant ($p < 0.05$) difference between sexes. # = Tendency ($p < 0.1$) difference between sexes.*

3.2 Ethical considerations and approvals

Prior to the study, the project was evaluated by the Regional Committees for Medical and Health Research Ethics and was found not to be within their mandate and could therefore be conducted without their approval. The study was also evaluated and approved by the Data Protection Official at the Norwegian Defence Research Establishment. The hierarchic system in the military relies on soldiers that follow orders from their superiors. A soldier in education is a subordinate member of this system, and can easily feel pressured to participate in the study when their superiors present the opportunity to participate. The importance of written consent was therefore specified to the candidates. It was also stressed that it would not have any negative consequence regarding their education or the upcoming FEX if they chose not to participate in the study. The commanders at FIH were instructed not to put any pressure on the soldiers to participate. The study was performed in compliance with the Declaration of Helsinki.

3.3 Experimental design

A test-battery consisting of measurements of muscle biopsies, body composition, blood samples, CMJ, maximal leg extension force, maximal leg extension power, maximal isokinetic and isometric force, aerobic and anaerobic performance was applied to measure the course of recovery after the FEX. In this master thesis, the following tests are included: muscle biopsy, body composition and maximal leg extension force. Therefore, the remaining tests will not be described in detail. The test-battery, with some variations, was applied at six time points during a six-week period (figure 3). The pre muscle biopsy was taken three weeks before the exercise to assure that the wound was completely healed before the exercise. The rest of the pre tests were carried out two weeks before the exercise. Thereafter, body composition was performed the day the participants returned from the FEX and the full test battery was performed after 24h, one week and two weeks after the FEX, with the exception of biopsies that were not taken after two weeks. Figure 4 presents a complete timeline of the study. The test-battery was executed in the same order at all time points. Body composition was measured in the morning (between 06:00 and 08:00) prior to breakfast, and muscle biopsies were taken between 06.00 and 13.00 while the physical test was performed 2-4h after the muscle biopsy. The only exception was the day they returned from the FEX when body composition was measured immediately after termination of the FEX. No other tests were done at this time point.



Figur 3: *Timeline of the study*

3.4 Field exercise "Cyber Endurance"

The field exercise "Cyber Endurance" lasted for approximately ten days. It is designed to test the candidates' physical and mental resilience in extreme situations in sub-optimal conditions. The content of the exercise was controlled solely by FIH and the scientists of this study had no influence on the content. Participation in the study did also not affect the content of the exercise or the soldier's ability to complete the FEX. The soldiers' objective during the FEX was mainly to resolve technological engineering tasks under realistic and demanding operational environment. They were mentally challenged as well as experiencing extreme stress and uncertainty.

From day one to three the soldiers ate as normal in the military camp, whereas during day four to nine, the soldiers received field rations and the average daily energy intake during this period was 900kcal/d for both men and women. They got about two to three hours of sleep each night. Accelerometers (ActiGraph wGT3X-BT, ActiGraph, Florida, USA), worn on the non-dominant wrist, were used to measure the activity energy expenditure during the FEX. Energy expenditure was calculated with the Freedson VM3 Combination algorithm. Basal metabolic rate was calculated with the formula BMR = 21.6 x LBM (kg) + 370 (Cunningham, 1991). Total energy expenditure was the sum of activity energy expenditure and basal metabolic rate. The FEX ended in the afternoon on day ten, and average energy expenditure was therefore estimated from measurements from day two to nine. The average energy expenditure per day during the

FEX was 5775 ± 558 kcal for men and 4717 ± 644 kcal for women.

3.5 Body Composition

Body composition was measured in the morning before breakfast (06:00 – 08:00) using bioelectrical impedance analysis (BIA) on an InBody 720 machine (Biospace Co., Seoul, Korea) according to the manufacturer's instructions. The participants were instructed to avoid eating, drinking and showering until the test was completed, and told to go to the toilet prior to the measurements. The participants stood upright the last five minutes before the measurement. These rigid standardizations were not possible at the post 0h time point when the BIA was administered immediately after the soldiers returned to base from the FEX. Participants performed all measurements in their underwear.

3.6 Test of physical performance

The soldiers completed the physical tests after a general warm-up and in the following sequence: CMJ, maximal leg extension force (Keiser), maximal leg extension power (Keiser), maximal isometric leg force (dynamometer), isokinetic leg force (dynamometer), aerobic performance test and Wingate test. Each test also included a specific warm-up, and strong verbal encouragement was given to the soldiers during tests that required maximal effort. Only the test of maximal leg extension force is described in detail.

3.6.1 **General warm-up**

The physical tests started with a ten minute long general warm-up that consisted of bicycling at low intensity and with two or three sprints during the last minutes.

3.6.2 **Maximal force**

Maximal force in the lower limbs was measured in a leg press machine where the resistance is regulated by air pressure (Keiser A-300, Fresno, CA, USA) with a protocol designed to measure maximal power and maximal force. The participants started with a specific warm up of eight repetitions with resistance of 80 N for women and 100 N for men. Thereafter, 1RM was estimated by gradually increasing resistance for each repetition until the participants reached their 1RM. The 1RM was approved when the maximal load had moved through a full range of motion, until the knees were fully extended. Both legs were assessed together. The participants had approximately a

minute rest in between the repetitions. Before starting the maximal power protocol, the participants had approximately five minutes rest.

Maximal force of the lower limb extensor muscles was measured using a protocol consisting of ten repetitions, where the estimated 1RM was used to define the perimeters of the test. For each repetition the resistance gradually increased until it reached a resistance just below 1RM. If the participants could execute more repetitions the resistance were further increased and they were instructed to continue the repetitions until they weren't able to perform more repetitions. The participants were instructed to execute the movement with the highest speed and force they could possibly perform on all the repetitions. Before the test began the participants had two test-extensions of their legs to ensure they understood how to produce the force as fast as possible. The machine calculated the power outcome and force at each repetition. Maximal force was identified as the single highest force achieved amongst all the lifts.

3.7 Muscle biopsy

Approximately 150mg resting muscle biopsy specimens were obtained from *m. vastus lateralis* while the participant was lying in a supine position. The area of the skin where the sample was taken was first disinfected by chlorhexidine and then local anesthesia was applied (Xylocaine adrenaline, 10 mg· ml−1+5 µl·ml−1, AstraZeneca, London, UK). A 1-2cm long and 15-20mm deep section was made in the skin and muscle fascia with a scalpel. A 6mm Bergström biopsy needle (Pelomi, Albertslund, Danmark) was inserted through the section and positioned primarily in distal direction. The biopsy needle was connected to a syringe creating a vacuum to make sure that the muscle tissue was sucked into the biopsy needle. Where a second extraction was necessary it was inserted into the same section and positioned in proximal direction. The muscle samples were rinsed in 9mg/ml sodium chloride and connective tissue, fat and blood was removed from the muscle samples. They were further cut into smaller pieces and weighed before they were placed in eppendorf tubes for different purposes, with ~50mg being sampled for immunoblotting and immediately frozen down by liquid nitrogen. The muscle samples were placed in a Styrofoam box with dry ice and transported by car to the Norwegian School of Sport Science the same day. Upon arrival, the muscle samples were stored in an ultra freezer at -80°C until further analyzes.

3.7.1 **Homogenizing**

A piece from the muscle samples (~50mg) were homogenized, for each of the 18 participants, by adding 1ml homogenizing buffer (TPER®, TissueProtein Extraction Reagent, cat#78510, Thermo Scientific Rockford, IL, USA), 20µl HaltTM protease, 10µl phosphatase inhibitor cocktail (cat#1861281, Thermo Scientific) and 10µl EDTA (Cat#1861274, Thermo Scientific, Rockford, IL, USA).

Each muscle sample was homogenized 2*3-5 seconds, or until all tissue was dissolved. After homogenizing the samples were shaken for 30minutes while placed in refrigerator with temperature 4°C. After this the samples were centrifuged by 10000G for ten minutes with temperature 4°C. The supernatant was transferred using pipettes into a 1,5ml tube and from this tube it was made 10 aliquots of 25µl each. The aliquots were then frozen down and stored at -80°C.

3.7.2 **Protein concentration**

Protein concentration was measured with RC / DC Protein assay kit (Bio-Rad, Cat # 5000121, Hercules, CA, USA). As standard protein, Bovine γ-globin was used with 0.125; 0.25; 0.5; 1; 1.5 µg · ml-1. The samples were diluted 1: 4 with ultra-pure water so that the protein concentration ended within the defined standard range. Triplicates with 5µl of each standard and 5µl of each sample were pipetted in a 96-well micro plate (Greiner Bio-One International AG, Kremsmünster, Germany). 25µl of reagent A · S (cat # 500-0113 and # 500-0115, Bio-Rad Laboratories Inc., USA) and 200µl of reagent B were then added to each well by multiples. The micro plate was incubated in a dark cabinet for a minimum of 15 and a maximum of 60 minutes, and then further analyzed by FLUOstar Omega (BMG LABTECH Gmbh, Offenburg, Germany) at 690 nm. Protein concentration (CV <10%) was calculated with Omega Software (V1.30).

3.7.3 **Western blot**

Electrophoresis and Western blot were performed on homogenized samples to investigate the total protein and phosphorylation status. The samples were diluted with ultra-pure water (dH_2O), LDS Sample buffer (4X, NP0008, NuPAGE, Invitrogen, USA) and Sample Reducing Agent (10X, NP0009, NuPAGE, Invitrogen, USA), to contain 30µg protein in each well. The samples were heated to 70°C for ten minutes before being pipetted into a 10-well NuPAGE 4-12% Bis-Tris Gel (NuPAGE, NP0321BOX,

Invitrogen, USA). 5µl weight marker (Protein Ladder PS 11, cat # 310005, GeneOn, Germany) was pipetted into the first and last well of each gel. Samples were run in duplicates and two gels were used for each person. Electrophoresis was run with cold running buffer of 200 volts for 66minutes (Power Pac 200, Bio-Rad, USA) or until the 5kDa weight mark was run out of the gel.

PVDF membranes (cat # 162-0177, Bio-Rad, CA, USA) were activated in methanol for 30 seconds (EMD Millipore Corporation, Billerica, MA, USA), then washed 1x30seconds and 1x2minutes in dH_2O before equilibrated in transfer buffer 15 minutes.

Proteins were transferred from gel to membrane with XCell II Blot Module (Invitrogen, CA, USA) at 30 volts for 90 minutes in cold transfer buffer. Further, the membranes were blocked in 5% skim milk solution (EMD Millipore Corporation, Billerica, MA, USA) in TBS-t (10X Tris Buffered Saline, Cat # 170-6435, Bio-Rad, CA, USA & 0.1% Tween® 20, cat # P7949-100 ml, Sigma Aldrich) for two hours at room temperature. After blocking, the membranes were cut into smaller portions based on the weight of the appropriate phosphorylated proteins. One gel was cut into $Ulk1^{Ser555}$ (140-150kDa), 4E-$BP1^{Thr70}$ (15-20kDa), $p70S6K^{Thr389}$ (70kDa), HADH (34kDa) and $Na^+K^+\beta1$ (50kDa), while the other gel was cut into $AMPK\alpha^{T172}$ (62kDa), COX-IV (16kDa) and CS (52kDa). They were then put into tubes and incubated with primary antibody (Table 6) at 4°C overnight on a grate board.

The next day, the membranes were washed with TBS-t for 15 minutes and then 3x5 minutes in TBS before incubated in secondary antibody diluted as described in table X with 1% skim milk solution (EMD Millipore Corporation, Billerica, MA, USA) and TBS-t. After incubation with secondary antibody, the membranes were again washed with the same procedure. Membranes were further incubated for 5 minutes with Chemiluminescent Substrate SuperSignal WestDura® (Extended Duration Substrate, Thermo Scientific, Cat # 34076, Rockford, IL, USA) before taking pictures using ChemiDoc™ MP Imaging System and analyzed with Image Lab™ Software (Bio- Rad Laboratories, Hercules, CA, USA).

After taking pictures of the phosphorylated proteins, the membranes where stripped in room temperature for ten minutes with Restore™ Western Blot Stripping Buffer (Ref 21059, Thermo Scientific, IL, USA). They were then quickly rinsed five times with

TBS before washing them 3x5 minutes with TBS. After this, the membranes were blocked in 5% skim milk solution in TBS-t for two hours at room temperature before . they were placed into tubes containing the appropriate antibody (Table 6) against total protein; ULK1 (150kDa), 4E-BP1 (15-20kDa), AMPKα (62kDa) and p70S6K (70kDa). The incubation were conducted at 4°C overnight on a grate board. The third day of the process, day 2 was repeated until pictures were taken. Figure 4 show representative protein bands for phosphorylated and total protein.

Figure 4: *Representative western blot protein bands. Phosphorylated proteins to the left and total protein content to the right. T1 indicates baseline, T2 is collected 24 hours after the completion of the exercise and T3 is collected one week after the exercise.*

Table 6: *Primary and secondary antibodies used for western blotting*

Antibody	Producer	Host animal	Dilution factor	Cat.no
p70 S6 Kinase	Cell Signaling	Rabbit	1:1000	2708
AMPKα	Cell Signaling	Rabbit	1:1000	2603
4E-BP1	Cell Signaling	Rabbit	1:1000	9452
ULK1 (Ser555)	Cell Signaling	Rabbit	1:1000	8054
Phospho-p70 S6 Kinase (Thr389)	Cell Signaling	Rabbit	1:1000	9234
Phospho-AMPKα (T172)	Cell Signaling	Rabbit	1:1000	2535
Phospho-4E-BP1 (Thr70)	Cell Signaling	Rabbit	1:1000	9455
Phospho-ULK1 (Ser555)	Cell Signaling	Rabbit	1:1000	5869
Anti-Citrate Synthetase	Abcam	Rabbit	1:4000	Ab96600
Anti-COX-IV	Abcam	Rabbit	1:2000	Ab16056
Anti-HADHSC	Abcam	Rabbit	1:8000	Ab154088
Na^+K^+-β1 (ATP1B1 monoclonal)	Thermo Scientific	Mouse	1:5000	MA3-930
Secondary Anti-mouse IgG (H+L) Antibody, HRP	Thermo Scientific	Goat	1:30000	31430
Secondary Anti-rabbit IgG HRP-linked Antibody	Cell Signaling	Goat	1:3000	7074

3.7.4 **Statistics**

All statistical analysis was completed in Prism 8 (GraphPad Prism, version 8.0.2 (159), GraphPad Software Inc., San Diego, CA, USA). A one-way Mixed Model ANOVA was used to investigate time changes for the group as a whole, and for men and women separately, for the phosphorylated proteins and the total protein content. A significant treatment effect was followed up with Bonferroni's multiple comparison tests of each groups' mean across different time points. Sex differences in percent change from pre-values at different time points was investigated by using a two-way Mixed Model ANOVA with sex as between subject factor, and time point during the study as within subject factor. A two-way Mixed Model ANOVA was also used to investigate changes over time, for the whole group, between sexes and for men and women separately, for each protein phosphorylated protein/total protein content (P/T), lean leg mass and maximal force. Sphericity was assumed in all tests, without any correction. A significant interaction within or between time and sex was followed up with Bonferroni's multiple comparisons test by comparing each group's mean across different time points within sexes, between sexes or for the whole group. Differences in anthropometrics before the study were investigated using independent sample t-tests. An alpha-level of 0.05 was used for all statistics. Values are mean ± standard deviation.

At T3, one value is missing for one man and one woman for each protein because of challenges related to the analyses.

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Article

Title: Sex differences in muscle function and myocellular response to a strenuous military field exercise

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Purpose: The purpose of this study was to investigate sex differences in the physiological and myocellular responses to a strenuous military field exercise (FEX), both acutely and in the recovery phase. **Methods:** Soldiers (F=8, M=10) from the Norwegian Defense Cyber Academy conducted a 10-day FEX. Body composition was measured by bioelectrical impedance before (T1) and 0 (T2-0), 1 (T2), 7 (T3) and 14 (T4) days after the FEX, whereas as maximal leg extension force was tested at T1, T2, T3 and T4. Skeletal muscle biopsies were obtained from *m. vastus lateralis* at T1, T2 and T3. Biopsies were analyzed for the following proteins involved in regulation of protein balance and metabolism by western blots: 4E-BP1, p70S6K, AMPKα, Ulk1, COX-IV, HADH, CS and Na^+K^+β1. **Results:** Lean leg mass increased with 4.4 ± 2.6% for men and women combined at T2-0 and returned to pre values at T3, with no sex difference. Men and women combined declined in maximal leg extension force with 9.2 ± 11% at T2 ($p<0.05$) and was still 8.8 ± 13 % reduced at T4 ($p<0.05$), with no sex differences. The ratio between phosphorylated and total AMPK ($AMPK_{P/T}$) increased by 39 ± 40% at T2 ($p<0.05$) and by 48 ± 47% at T3 ($p<0.001$). At T3, we also report a 46 ± 49% increase in the phosphorylation of $AMPK^{Thr172}$ ($p<0.01$). We observed tendencies for sex difference in the relative change from T1 for 4E-BP1 ($p=0.082$) and Na^+K^+β1 ($p=0.065$) at T2 and in p70S6K ($p=0.057$) at T3. At T2, there were a significant different in the change in Ulk1 ($p<0.05$), where women decreased 22 ±15 % and men increased 16 ± 17%. Na^+K^+β1 decreased by 7 ± 12% ($p<0.05$) at T2 and 12 ± 13% ($p<0.05$) at T3. COX-IV decreased 15 ± 43% at T2 ($p<0.05$), and was recovered at T3, whereas CS decreased 18 ± 21% ($p<0.05$) at T3. There were no significant changes in HADH. **Conclusion:** A strenuous military field exercise resulted in similar reductions in maximal force for men and women together with increased catabolism and/or inhibited anabolism, which lasted for at least one week. There was a sex difference in the change in protein levels of Ulk1, but this was not related to any difference in reduction of maximal force. The results indicate that there may be sex differences in the myocellular response to a military field exercise, but further investigations are needed. **Key words**: SOLDIER, PHYSICAL PERFORMANCE, RECOVERY, PROTEIN SYNTHESIS, AUTOPHAGY

Introduction

A strenuous military field exercise (FEX) exposes soldiers of extreme stress, including energy deficit, sleep deprivation and physical strain (1, 2). Reductions of body mass and lean body mass (LBM) during FEXs of varying durations and intensities have previously been reported (3-7) and some argue that having more than 10% loss in body mass results in huge decrements in physical performance (1, 2, 4, 8). Several studies show detrimental effects on physical performance after FEXs, including reductions in maximal muscle strength (4-6) and explosive muscle strength (3-6, 9-12), which is known as muscle fatigue (13). However, in many of these studies the reduction in body mass is less than 10% (5, 9-12) and the relationship between changes in body composition and physical performance after FEX is still unclear (5, 14, 15). Knowledge of the recovery process of muscle force-generating capacity is of high importance to combat-personnel, because the majority of physical tasks that soldiers conduct are dependent on strength and power performance (15). The muscle fatigue can potentially last for several weeks, which may be caused by loss of skeletal muscle or changes in muscle biochemistry (5, 8). The recover process of physical performance after a strenuous FEX has, on the other hand, been less investigated. There are, to our knowledge, only three studies reporting on the physical recovery (3, 5, 12), and no study reporting on recovery of myocellular changes.

Muscle mass is one of the main determinants of muscle force-generating capacity (16). In order to recover lost muscle mass the protein balance needs to be positive, which is determined by the rates of protein synthesis and protein breakdown (17). The mechanistic target of rapamycin complex 1 (mTORC1) is the key-mediator of the protein synthesis. It stimulates translation by activation of ribosomal protein S6 kinase (S6K1) and increased phosphorylation of elongation factor-4E binding protein 1 (4E-BP1) (18). Protein breakdown is mainly regulated in two systems, the autophagy system and the ubiquitin-proteasome system (17). The unc-51 like autophagy activating kinase 1 (Ulk1) protein complex initiates the autophagy process (11). Ulk1 is activated when phosphorylated by 5' AMP-activated protein kinase (AMPK) and inhibited when phosphorylated by mTORC1, which provide the cells with a mechanism to respond to a wide range of stimuli (19). One bout of exercise will stimulate both protein synthesis and breakdown. However, if food is consumed, then the protein synthesis will be

stimulated in a greater manner than protein breakdown and thus result in a positive protein balance (20). The increased response in protein synthesis to a single bout of exercise is thought to mainly be reflected by activation of the existing translational machinery, whereas longer periods of exercise has the potential to increase the total translational capacity within the muscles (21). Different exercise stimuli also seem to affect which proteins are being synthesized in the recovery period. Prolonged low-intensity stimulation, like endurance exercise, increases the mitochondrial biogenesis, causing oxidative and fatigue resistant muscle fibers. Prolonged resistance exercise, on the other hand, increases the synthesizing of myofibrillar proteins, causing hypertrophy (20). The current observations indicate that a strenuous FEX causes a negative protein balance, primarily affected by increased protein breakdown (9, 11). Negative energy balance in muscle tissue is a signal to degrade proteins in order to provide energy and amino acids to other tissue (17), which may be the reason for the observed increase in protein breakdown. A second reason could be intensified myocellular need to degrade or remove damaged or dysfunctional proteins (17).

The majority of military studies have been conducted solely on men. However, the number of women serving and enlisting is increasing. In general, women are inferior to men when it comes to most aspects of physical performance (13, 22). However, a recent study reports that both men and women decrease similar in relative physical performance (12), even if the men experience a greater loss of fat-free mass (FFM) after FEX (12, 14). Interestingly, it is indicated that during the recovery phase the women may return faster than men to baseline values in FFM and physical performance (12). These sex differences is thought to occur because women have lower resting metabolism, they are better able to tolerate changes in body composition, and they utilize fat reserves better, which promotes protection against lean body mass (LBM) losses (5, 7, 8, 23). To our knowledge only one study have investigated sex differences in how physical performance is affected by and recovers from a demanding FEX (12), and there are no studies on possible sex differences in the myocellular recovery after a FEX.

The aim of this study was therefore to investigate sex differences in the physiological response in lean leg mass (LLM) and force-generating capacity, and myocellular responses involved in the regulation of protein balance and energy production, to a

strenuous FEX, both acutely and in the recovery phase. We hypothesized that the responses to a FEX would be similar for the sexes, and that women would show a faster recovery compared to men. Furthermore, we hypothesized that regulators of protein balance would shift to a more negative state right after the FEX, and that it would increase during the recovery phase.

Material and Methods

Participants

Eighteen cyber engineering soldiers from a larger troop, eight women (21.4 ± 1.4 years, 65.4 ± 12.3 kg), and ten men (20.5 ± 0.5 years, 76.7 ± 6.4 kg), that was going to participate in a FEX as a obligatory part of their studies, volunteered to participate in this study. Participation in the study did not affect the content of the FEX or the soldier's ability to complete the FEX. After information was given verbally and in writing, all 18 participants gave their written informed consent. The study was evaluated by the Regional Committees for Medical and Health Research Ethics and approved by the Data Protection Official at the Norwegian Defence Research Establishment, and was performed in accordance with the principles outlined in the Declaration of Helsinki.

Experimental design

The measurements and test were performed two and three weeks prior to the FEX (T1), when the soldiers retuned to base after the FEX (T2-0), the day after the FEX (T2), one (T3) and two weeks (T4) after the FEX, The timeline and which tests that were included in different testing days are shown in figure 1. Body composition was performed in the morning before breakfast (between 06:00 and 08:00) at all times. Muscle biopsies were taken between 07:00 and 13:00 at T1, T2 and T3 and the physical performance tests were performed between 09:00-15:00 at T1, T2, T3 and T4. There was a 2-4 hours gap between the muscle biopsies and physical performance test at T2 and T3. Participants performed the tests at the same time of day at each testing day (within one hour).

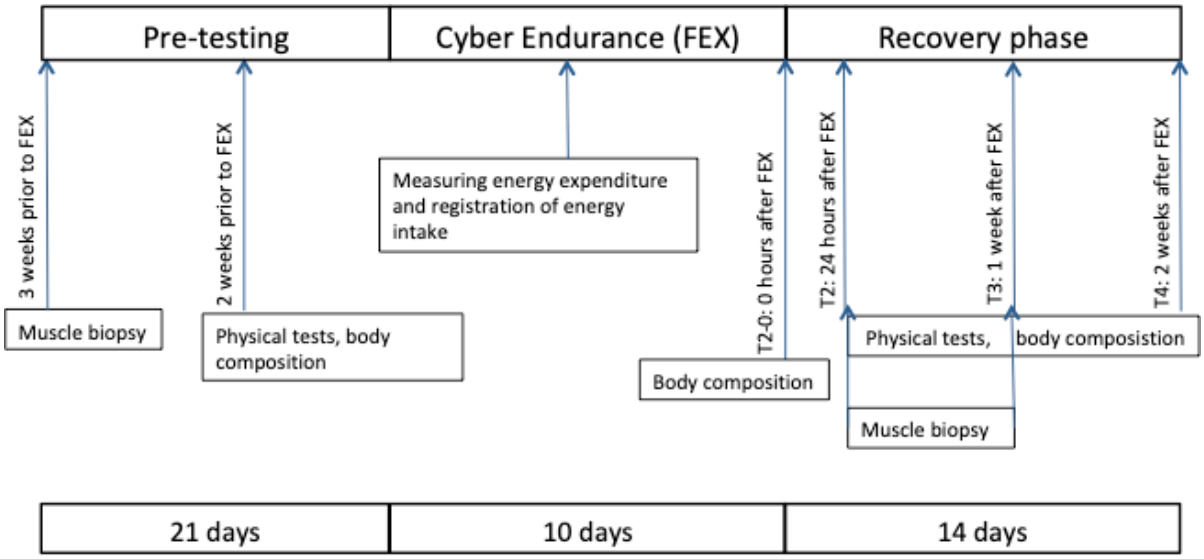

Figure 1: Timeline of the study

The Cyber Endurance exercise

The FEX used in this study is called "Cyber Endurance" and lasted for ten days. It consisted of high degree of various types of physical activity while performing technical tasks and being exposed to sleep deprivation and low energy intake. The soldiers' objective during the FEX was mainly to resolve technological engineering tasks under realistic and demanding operational environment. The content of the exercise was controlled solely by the Norwegian Defense Cyber Academy and the scientists of this study had no influence on the content.

From day one to three the soldiers ate as normal in the military camp, whereas during day four to nine, the soldiers received field rations and the average daily energy intake during this period was 900 kcal/d for both men and women. They got approximately two to three hours of sleep each night. Accelerometers (ActiGraph wGT3X-BT, ActiGraph, Florida, USA), worn on the non-dominant wrist, were used to measure the activity energy expenditure during the FEX. The energy expenditure was calculated with the Freedson VM3 Combination algorithm in the Actilife software (ActiGraph wGT3X-BT, ActiGraph, Florida, USA). Basal metabolic rate was calculated with the formula BMR = 21.6 **x** LBM (kg) + 370 (24). Total energy expenditure was the sum of activity energy expenditure and basal metabolic rate. The FEX ended in the afternoon on day ten, and average energy expenditure was therefore estimated from measurements

from day two to nine. The average energy expenditure per day during the FEX was 5775 ± 558 kcal for men and 4717 ± 644 kcal for women.

Body composition

Body composition was measured in the morning before breakfast (between 06.00 and 08.00) using bioelectrical impedance analysis (BIA) on an InBody 720 machine (Biospace Co., Seoul, Korea) according to the manufacturer's instructions. The participants were instructed to avoid eating, drinking and showering until the test was completed, and told to go to the toilet prior to the measurements. The participants stood upright the last five minutes before the measurement. These rigid standardizations were not possible at T2-0 when the BIA was administered immediately after the soldiers returned to base from the FEX. Participants performed all measurements in their underwear.

Muscle strength

A general warm-up of bicycling at low intensity was conducted for ten minutes, with two or three sprints during the last minutes. Maximal force in the lower limbs was measured in a leg press machine where the resistance is regulated by air pressure (Keiser A-300, Fresno, CA, USA) as part of a maximal force and maximal power protocol. Both legs were assessed together. The participants had a specific warm up of eight repetitions with resistance of 80 N for women and 100 N for men and an estimation of one repetition maximum (1RM) before the maximal force and power protocol. The 1RM was estimated by increasing the resistance gradually for each repetition that followed until the participants reached the maximum load they could lift, in their own speed and through a full range of motion, which was then defined as their 1RM. The participants had approximately a minute rest in between the repetitions. Before starting the test protocol the participants had approximately five minutes rest.

Maximal force of the lower limb extensor muscles was measured using a protocol of ten repetitions, where the estimated 1RM was used to define the perimeters of the test. For each repetition the resistance gradually increased until it reached just below 1RM. If the participants could execute more repetitions they were instructed to continue the

repetitions until they weren't able to perform more repetitions. The participants were instructed to execute the movement with the highest speed and force they could possibly perform on all the repetitions. Before the test began the participants had two test-extensions of their legs to ensure they understood how to produce the force as fast as possible. The machine calculated the power outcome and force at each repetition and maximal force was identified as the single highest force achieved amongst all the lifts.

Muscle biopsy

Approximately 150mg resting muscle biopsy specimens were extracted from *m. vastus lateralis* while the participant was lying in a supine position. The skin was disinfected by chlorhexidine before applying local anesthesia using 2–3ml Xylocaine (Xylocaine adrenaline, 10 mg· ml−1+5 µl·ml−1, AstraZeneca, London, UK). A 6mm Bergström biopsy needle (Pelomi, Albertslund, Danmark) was inserted through the section and positioned primarily in distal direction and where a second extraction was necessary it was inserted into the same section and positioned in proximal direction. The muscle samples were rinsed in 9mg/ml sodium chloride and connective tissue, fat and blood was removed. They were further cut into smaller pieces and weighed before they were placed in eppendorf tubes for different purposes, with ~50mg being sampled for immunoblotting and immediately frozen down by liquid nitrogen. Once the muscle samples were returned to the Norwegian School of Sport Science the same day, they were stored in an ultra freezer at -80°C until further analyzes.

Immunoblotting

About 50mg muscle was homogenized by adding 1ml homogenizing buffer (TPER®, TissueProtein Extraction Reagent, cat#78510, Thermo Scientific Rockford, IL, USA), 20µl HaltTM protease, 10µl phosphatase inhibitor cocktail (cat#1861281, Thermo Scientific) and 10µl EDTA (Cat#1861274, Thermo Scientific, Rockford, IL, USA). Samples were subsequently shaken for 30minutes while placed in refrigerator and later centrifuged by 10000G for ten minutes, both processes holding 4°C.

Electrophoresis and Western blot were performed on the homogenized samples to investigate the total protein and phosphorylation status. The samples were diluted with

ultra-pure water (dH2O), LDS Sample buffer (4X, NP0008, NuPAGE, Invitrogen, USA) and Sample Reducing Agent (10X, NP0009, NuPAGE, Invitrogen, USA), to contain 30µg protein in each well. The samples were heated to 70°C for ten minutes before being pipetted into a 10-well NuPAGE 4-12% Bis-Tris Gel (NuPAGE, NP0321BOX, Invitrogen, USA). 5µl weight marker (Protein Ladder PS 11, cat # 310005, GeneOn, Germany) was pipetted into the first and last well of each gel. Samples were run in duplicates and two gels were used for each person. Electrophoresis was run with cold running buffer of 200 volts for 66 minutes (Power Pac 200, Bio-Rad, USA) or until the 5kDa weight mark was run out of the gel

PVDF membranes (cat # 162-0177, Bio-Rad, CA, USA) were activated in methanol for 30 seconds (EMD Millipore Corporation, Billerica, MA, USA), then washed 1x30seconds and 1x2minutes in dH2O before equilibrated in transfer buffer 15 minutes.

Proteins were transferred from gel to membrane with XCell II Blot Module (Invitrogen, CA, USA) at 30 volts for 90 minutes in cold transfer buffer. Then, the membranes were blocked in 5% skim milk solution (EMD Millipore Corporation, Billerica, MA, USA) in TBS-t (10X Tris Buffered Saline, Cat # 170-6435, Bio-Rad, CA, USA & 0.1% Tween® 20, cat # P7949-100 ml, Sigma Aldrich) for two hours at room temperature. After blocking, the membranes were cut into smaller portions based on the weight of the appropriate phosphorylated proteins. They were then put into tubes and incubated with primary antibodies for Ulk1^{Ser555}, 4E-BP1^{Thr70}, p70S6K^{Thr389}, Hydroxyacyl CoA dehydrogenase (HADH), AMPKα^{T172}, Cytochrome c oxidase or complex IV (COX-IV) and Citrate Synthase (CS) from Cell Signaling (USA) and Na^+K^+β1 from Thermo Scientific (USA) at 4°C overnight on a grate board.

The next day, the membranes were washed with TBS-t for 15 minutes and then 3x5 minutes in TBS before incubated in appropriate secondary antibody against rabbit from Cell Signaling (USA) and against mouse from Thermo Scientific (USA) diluted with 1% skim milk solution (EMD Millipore Corporation, Billerica, MA, USA) and TBS-t. After incubation with secondary antibody, the membranes were again washed with the same procedure. Membranes were then incubated for 5 minutes with Chemiluminescent Substrate SuperSignal WestDura® (Extended Duration Substrate, Thermo Scientific, Cat # 34076, Rockford, IL, USA) prior to taking pictures with the ChemiDoc ™ MP

Imaging System and analyzed with Image Lab ™ Software (Bio- Rad Laboratories, Hercules, CA, USA).

After taking pictures of the phosphorylated proteins, the membranes where stripped in room temperature for 10 minutes with Restore™ Western Blot Stripping Buffer (Ref 21059, Thermo Scientific, IL, USA). They were then quickly washed 5 times with TBS, before washing them 3x5 minutes with TBS. Then, the membranes were blocked in 5% skim milk solution in TBS-t for two hours at room temperature. After blocking, the membranes placed into tubes containing the primary antibody against total protein; Ulk1, 4E-BP1, AMPKα and p70S6K from Cell Signaling (USA). The incubation were conducted at 4°C overnight on a grate board. The third day of the process, day 2 was repeated until pictures were taken.

Statistical analysis

All statistical analysis was completed in Prism 8 (GraphPad Prism, version 8.0.2 (159), GraphPad Software Inc., San Diego, CA, USA). A one-way Mixed Model ANOVA was used to investigate time changes for the group as a whole, and for men and women separately, for the phosphorylated proteins and the total protein content. A significant treatment effect was followed up with Bonferroni's multiple comparison tests of each groups' mean across different time points. Sex differences in percent change from pre-values at different time points was investigated by using a two-way Mixed Model ANOVA with sex as between subject factor, and time point during the study as within subject factor. A two-way Mixed Model ANOVA was also used to investigate changes over time, for the whole group, between sexes and for men and women separately, for each protein phosphorylated protein/total protein content (P/T), lean leg mass and maximal force. Sphericity was assumed in all tests, without any correction. A significant interaction within or between time and sex was followed up with Bonferroni's multiple comparisons test by comparing each group's mean across different time points within sexes, between sexes or for the whole group. Differences in anthropometrics before the study were investigated using independent sample t-tests. An alpha-level of 0.05 was used for all statistics. Values are mean ± standard deviation.

At T3, one value is missing for one man and one woman for each protein because of challenges related to the analyses.

Results

Body mass and body composition

The body mass for the sexes combined decreased 5.6 ± 1.8% ($p < 0.0001$) from T1 to T2-0 (table 1) and did not recover after two weeks of rest (-2.5 ± 2.2%, $p < 0.0001$). The same pattern was displayed for fat mass (FM), being reduced 34.3 ± 11.0% at T2-0 and still reduced after two weeks of rest (-12.5 ± 7.7%, $p < 0.0001$). LBM decreased for the sexes combined from T1 to T2-0 with -1.5 ± 2.1% ($p < 0.01$), tended to be different at T2 (-1.2 ± 2.0%, $p = 0.054$), and was back to baseline at T3.

There were no significant differences between the sexes in the relative change of body mass, LLM, LBM or FM. Men had a significantly higher LLM compared to the women at all time points (Figure 2a, $P < 0.0001$). LLM increased with 4.7 ± 3.4% and 4.1 ± 2.0% during the FEX for women and men respectively (figure 2b, $P < 0.001$). At T3 and T4 LLM was back to baseline.

Table 1: *Changes in body mass and body composition for the sexes combined during the study*

	T1	T2-0	T2	T3	T4
BM (kg)	71.7 ± 10.8	67.6 ± 9.9 *	68.0 ± 10.0 *	69.7 ± 9.9 *	69.8 ± 10.1 *
LBM (kg)	34.0 ± 5.9	33.5 ± 5.8 *	33.5 ± 5.6 #	34.1 ± 5.7	33.7 ± 5.7
FM (kg)	11.8 ± 5.0	8.2 ± 4.8 *	8.6 ± 4.6 *	9.7 ± 4.6 *	10.4 ± 4.8 *

*Values are mean ± standard deviation. * = Significant ($p < 0.05$) difference from T1. # = Tendency ($p < 0.1$) difference from T1. BM= Body mass. LBM= Lean body mass. FM= Fat mass. T2-0= right after FEX. T2= one day after FEX. T3= one week after FEX. T4= two weeks after FEX*

Strength performance

Men had a higher maximal leg extension force at all time points compared to the women, but this was only significantly different at baseline (figure 2c, $P < 0.05$). Both sexes combined reduced maximal force after the FEX with 9.2 ± 11.0% ($P < 0.01$), and

was still 10.0 ± 9.5% (P < 0.001) and 8.8 ± 12.6% (P < 0.01) reduced at T3 and T4, respectively. These reductions occurred because the men reduced maximal force by 11.6 ± 10.5 % (P < 0.001) during the FEX that was still -11.8 ± 10.3% (P < 0.001) and -12.5 ± 10.0% (P < 0.001) at T3 and T4, respectively. No significant changes occurred in the women, but the relative changes in maximal force were not different between sexes.

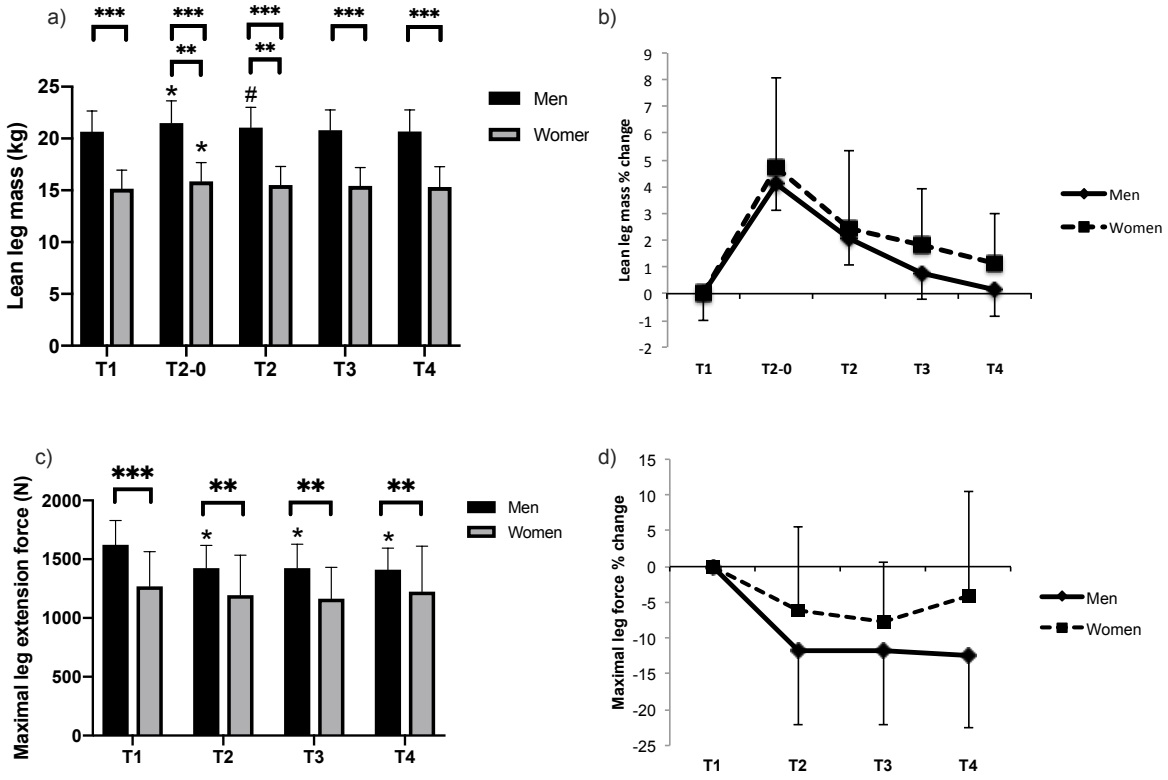

Figure 2: *a) Leg lean mass during the study period on both men (black bars) and women (gray bars). b) Percent change in lean leg mass in both men (solid line) and women (dotted line). c) Maximal leg extension force during the study period on both men (black bars) and women (gray bars). d) Percent change in maximal leg extension force in both men (solid line) and women (dotted line). Values are shown mean ± SD. * = significant change from T1 within sex. ** = significant change from T1 for the whole group. *** = significant difference between sexes. # = tendency for change from T1 within sex. Significancy= p<0.05. Tendency= p<0.10.*

Regulators of protein synthesis and protein breakdown

The p70S6K-protein levels and the phosphorylation status of p70S6K^{Thr389} did not change for men, women or the sexes combined from T1 to T2 (figure 3a). However, men tended to have reduced p70S6K-protein levels at T3 compared to T1 (-8.5 ± 19.3%, P = 0.08), whereas women had non-significantly increased

(10.7 ± 28.5%, P = 0.79), resulting in a tendency (P = 0.057) for sex difference in the relative change at T3 (figure 3c).

The total levels of 4E-BP1-protein did not change for the men and women combined during the FEX, but tended to increase 14.5 ± 32.5% compared to T1 at T3 (figure 3g, p = 0.058). At T2 men had significantly increased 4E-BP1-protein levels (27.3 ± 41.9%, p < 0.05), whereas women had a non-significant reduction (-7.5 ± 26.6%, P > 0.99), which led to a tendency to a difference between sexes (figure 3i, P = 0.082). The phosphorylation status of 4E-BP1^{Thr70} and 4E-BP1$_{P/T}$ did not change for the sexes combined or separately during the study.

The total levels of Ulk1-protein, the AMPK-mediated phosphorylation of Ulk1^{Ser555} and Ulk1$_{P/T}$ did not change for men and women combined or separately during the study. However, at T2, the women had a tendency for reduction in Ulk1-protein levels (-22.5 ± 15.6%, P = 0.097) compared to T1, whereas men had a non-significant increase (13.7 ± 23.5%, P = 0.55), which resulted in a significant sex difference in the relative change at T2 (figure 4c, P < 0.05). Women also tended to have increased Ulk1$_{P/T}$ at T3 (P = 0.095).

The AMPK-protein levels did not change during the study, but a significant 45.6 ± 48.6 % increase was observed in the phosphorylation of AMPKThr172 for men and women combined (P < 0.01) and for the men (51.1 ± 38.9%, P < 0.05) at T3 compared to T1. AMPK$_{P/T}$ increased significantly 39.0 ± 40.6% for men and women combined at T2 (Figure 4j, P < 0.05) and for men separately with 45.7 ± 42.6% (p < 0.05), with no sex difference. At T3, AMPK$_{P/T}$ was still significantly increased 48.2 ± 46.6% for men and women combined (P < 0.001), and 52.9 ± 30.7% for men separately (p < 0.001), with no sex difference.

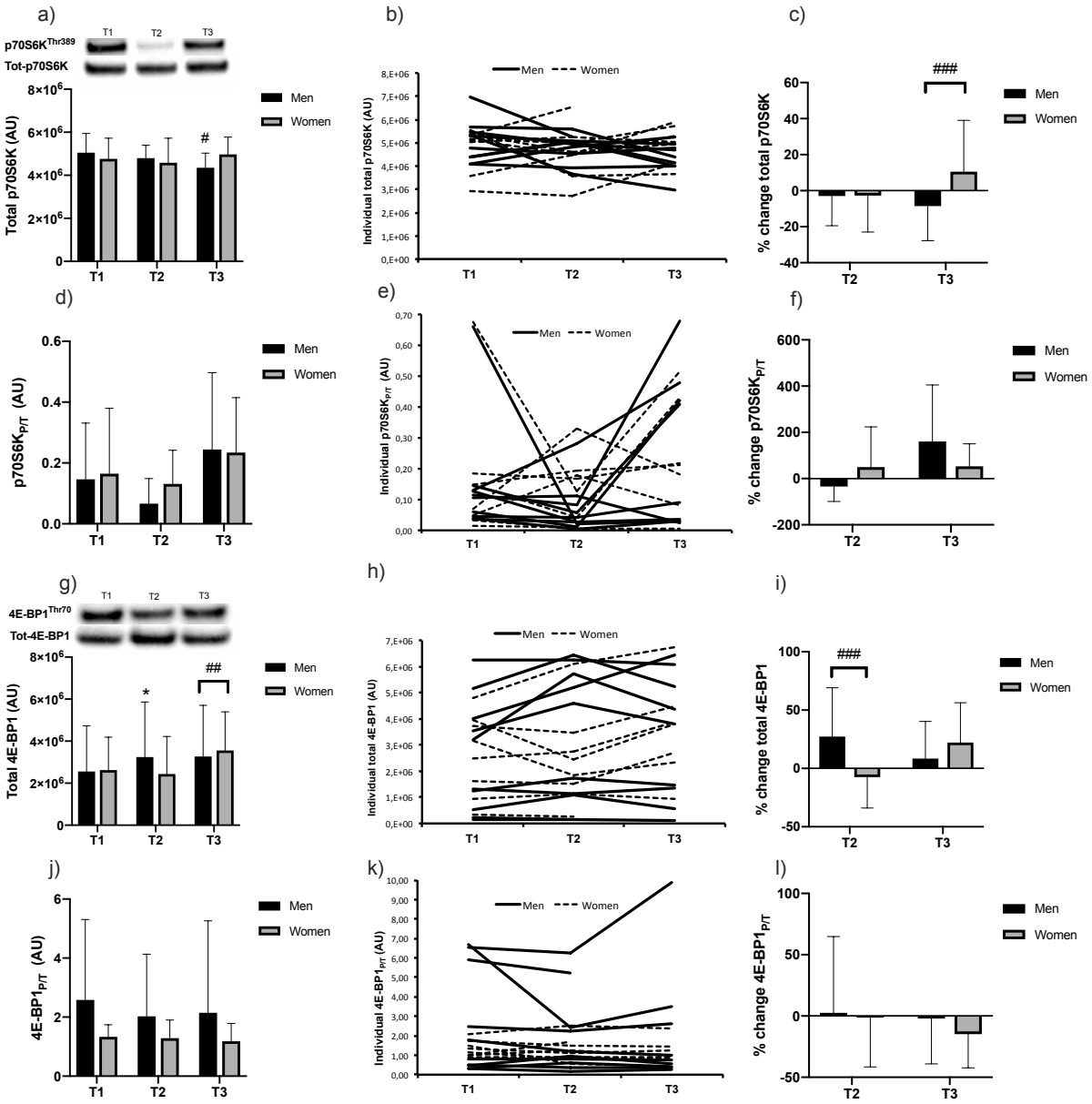

Figure 3: *Changes in total protein levels and phosphorylated/total protein (P/T) for p70S6K (a-f) and 4E-BP1 (g-l). Representative blots are shown above the graph of total protein content. Values are shown as mean ± SE. * = significant change from T1 within sex. # = tendency for change from T1 within sex. ## = tendency for change from T1 for the whole group. ### = tendency for difference between sexes. Significancy= p<0.05. Tendency= p<0.10.*

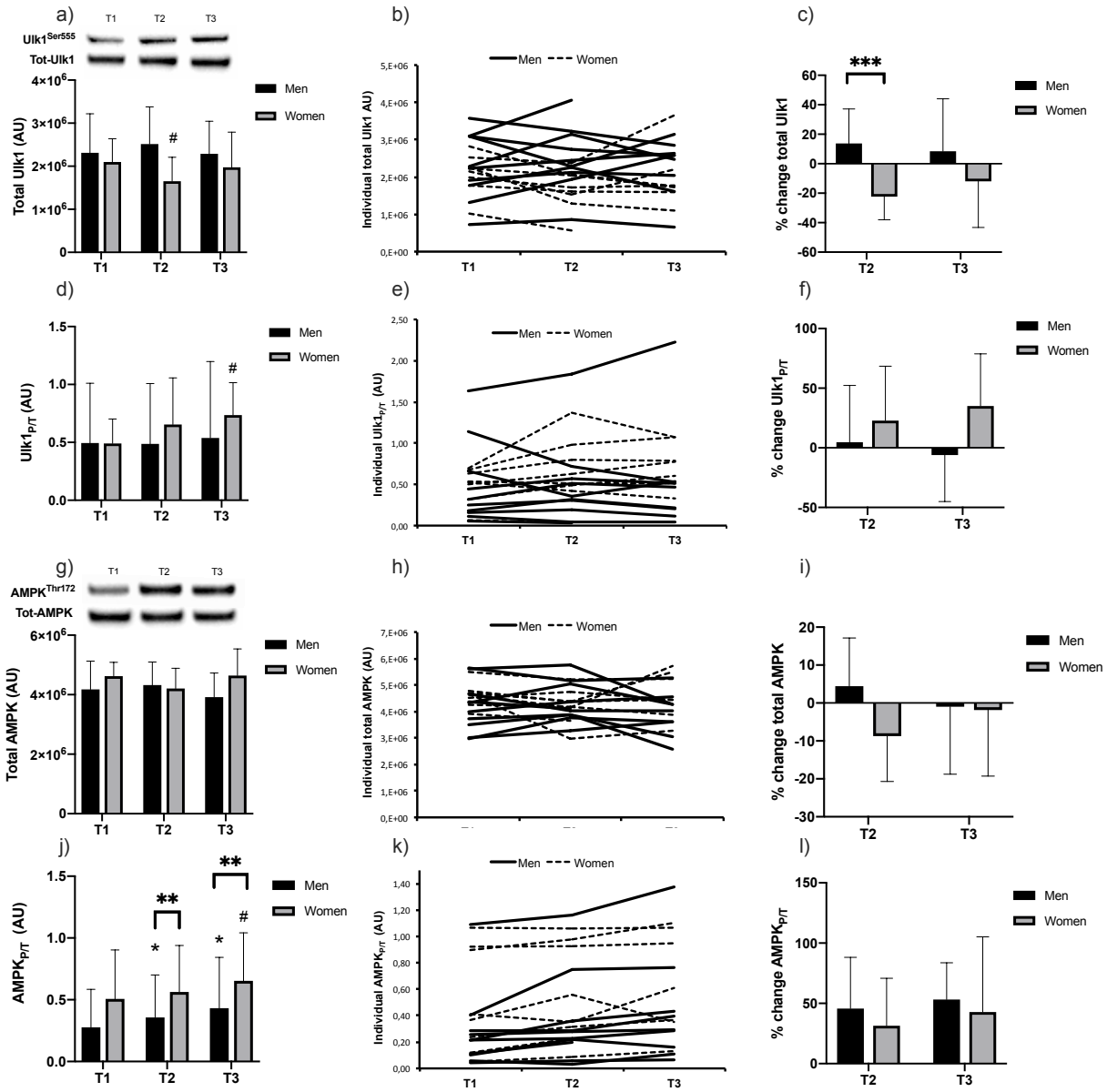

Figure 4 *Changes in total protein levels and phosphorylated/total protein (P/T) for Ulk1 (a-f) and AMPK (g-l). Representative blots are shown above the graph of total protein content. Values are shown as mean ± SE. * = significant change from T1 within sex. ** = significant change from T1 for the whole group. *** = significant difference between sexes. # = tendency for change from T1 within sex. Significancy= p<0.05. Tendency= p<0.10.*

Mitochondrial proteins (COX, CS, HADH)

There was a significant 14.7 ± 43.4% decrease in COX-IV levels for the men and women combined and for the women separately from T1 to T2 (figure 5a, $P < 0.05$), as well as a tendency for 12.1 ± 41.7% reduction at T3 for the sexes combined ($P = 0.061$). There were no significant differences between the sexes.

There were no changes in CS levels during the FEX for the sexes separately or combined, or between the sexes. However, at T3, men and women combined had a significant 17.9 ± 20.9% decrease (figure 5d, $P < 0.05$).

There were no significant changes in HADH levels.

Na^+K^+

$Na^+K^+\beta1$ levels significantly decreased 6.9 ± 12.6% for the sexes combined ($P < 0.05$) and 14.4 ± 9.2% for the women separately ($P < 0.01$) from T1 to T2, while no significant change occurred in the men (-0.8 ± 12.0%, $P > 0.99$). This change tended to be different between sexes ($P = 0.061$). At T3 $Na^+K^+\beta$ levels were still significantly reduced 11.9 ± 13.4% for the sexes combined ($P < 0.001$) and 14.3 ± 13.4% for the women ($P < 0.01$) and also significantly reduced 10.0 ± 13.9% for the men ($P < 0.05$). There were no differences between the sexes.

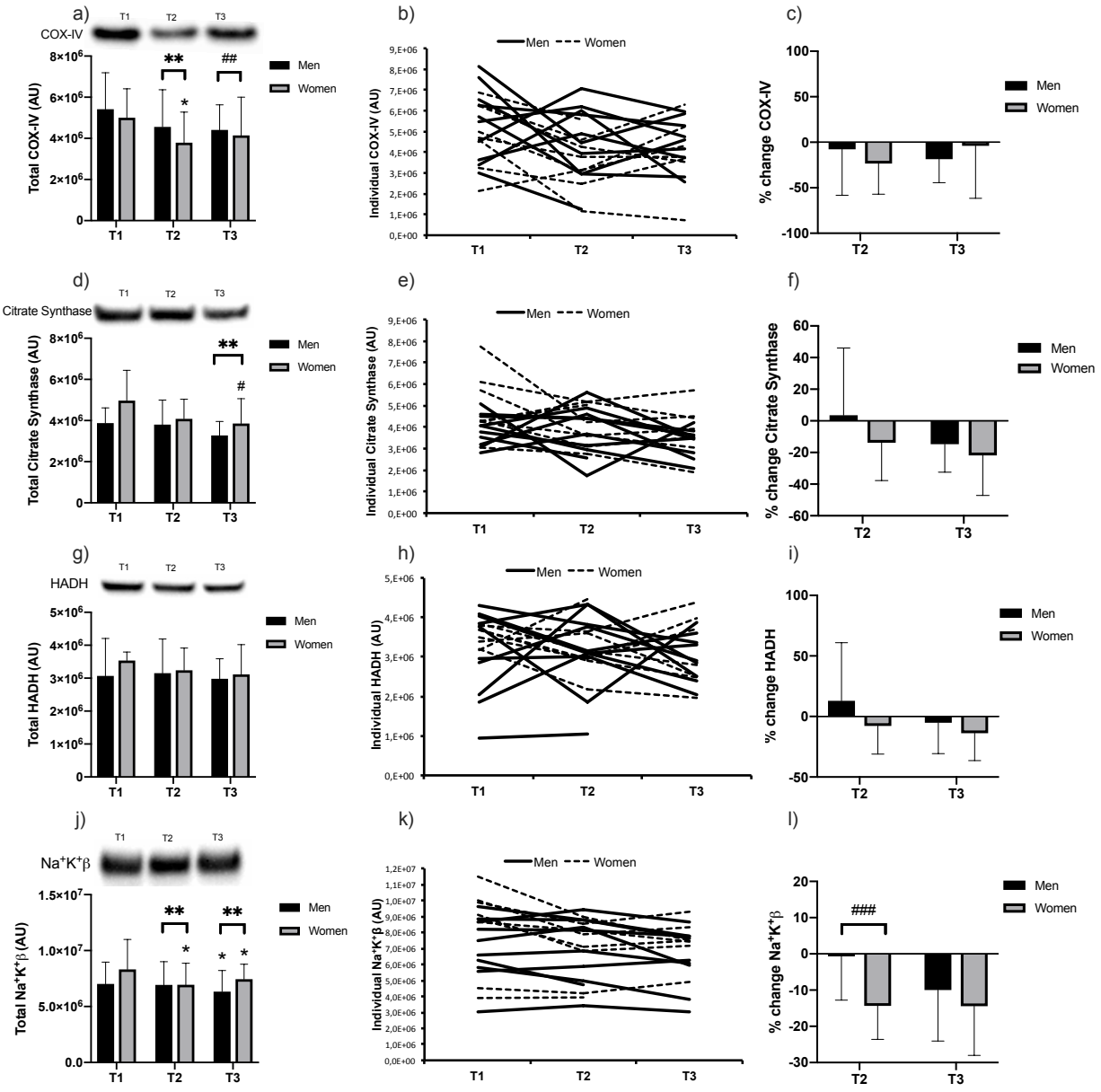

Figure 5: *Changes in protein levels for COX-IV (a-c), Citrate Synthase (d-f), HADH (g-i) and Na^+K^+ (j-l). Absolute values for the sexes and % changes are shown as mean ± SE. * = significant change from T1 within sex. ** = significant change from T1 for the whole group. *** = significant difference between sexes. # = tendency for change from T1 within sex. ## = tendency for change from T1 for the whole group. ### = tendency for difference between sexes. Significancy= p<0.05. Tendency= p<0.10.*

Discussion

The main aim of this study was to investigate sex differences in the acute physiological and myocellular responses to a strenuous FEX and how these recovered during the first two weeks after the FEX. Our study is the first study to investigate sex difference in myocellular responses in the recovery phase of a FEX. The main findings were that

$AMPK_{p/t}$ increased after FEX and further increased after one week of rest. Ulk1-protein levels did not change, but we found a sex difference in the relative change in Ulk1-protein levels right after FEX because of a non-significant increase in men and non-significant reduction in women. The regulators of protein synthesis did not change during the study. $Na^+K^+\beta 1$ were slightly decreased right after FEX and also after one week of rest, whereas a decreased COX right after FEX was recovered after one week of rest. CS did not change during the FEX, but decreased significantly after one week of rest. The LLM increased after FEX and returned to baseline values after one week of rest, whereas maximal leg extension force decreased after FEX and was not recovered after two weeks of rest.

Changes in lean leg mass

LLM for men and women combined increased after FEX, and gradually decreased to baseline values after one week of recovery, without any sex difference in the relative change. This is not in accordance with what most studies report, which is that soldiers experience a loss of LBM or FFM after FEXs (3, 5, 12, 14), and that this loss gradually increases back to baseline within one week (3, 5, 12). However, there are also some studies that show a possible preservation of FFM or even small increases in FFM (7, 25).

The reason for these discrepancies are unclear, but might be related to methodological reasons. For example, most studies report total LBM and not for different body parts, and we cannot exclude a reduction in other body parts. In fact, we also found a reduction in the total LBM and this is therefore similar to most studies. Another option is that the increased LLM is a response to damaging exercise during the FEX, causing muscle damage and swelling (26), which might affect the measurement. Furthermore, the increase in LLM was significant for women and for men straight (T2-0) after the FEX, but not the day after (T2). At T2-0 the BIA was not performed under standardized conditions and the results should therefore be interpreted with caution since there is a pre-requisite to abstain from exercise before a measurement (27). This implies that the initial increase observed in LLM may not be correct. However, the increase at T2 was, nonetheless, significant for the sexes combined.

We cannot exclude if, for example, carrying heavy backpacks or other heavy lifting during the FEX had a training effect in the legs and not the whole body. However, the loss of body mass in our study was consistent with what is most common for this duration of FEX (5, 7, 28), and we should therefore theoretically have seen a decrease in LLM as a consequence of this. On the other side, losses in body mass during FEX primarily consist of losses in FM, which was also the case in the current study, and it has been suggested that having FM over a certain threshold protects against loss of muscle mass (5, 12), and our soldiers were within normal range of FM (22). Therefore, losses of LBM during FEX may vary due to different total energy expenditure during the FEX, initial FM or different procedures for measuring and reporting body mass and body composition (7, 14).

Changes in maximal leg extension force

Maximal leg extension force decreased for men and women combined after FEX, which is consistent with results from other studies on the responses of a strenuous FEX (1, 3, 5, 11, 12). This decrease did not recover within two weeks of rest, which is different from the full recovery after two weeks rest that Hamarsland et al (5) found in maximal isometric leg press after a strenuous FEX (5). On the other hand, reduced CMJ performance did not recover within two weeks in that study, which is also reported by a second Norwegian study of responses in Special Forces after a strenuous FEX (5, 12). Seen together, these previous studies indicate that factors related to maximal muscle power are affected by strenuous FEX in a greater manner, and recover slower, than factors related to maximal muscle force. Our maximal leg extension test was performed in a manner where the soldiers were instructed to produce force as quickly as possible and may therefore be more related to factors affecting maximal power than maximal isometric force. In this regard the result is in accordance with previous findings. Previously, reduced LBM has been suggested to be an important reason for reduced physical performance seen after FEXs (1) and the increase in LLM together with reduced force is therefore surprising. However, as discussed above, the increase in LLM might have been related to methodological issues. LLM and LBM is, nonetheless, recovered within one week of rest for, and may therefore not explain the maintained decrease in maximal leg extension force for the sexes combined after two weeks of recovery. Notably, the body mass and FM was still reduced after two weeks of rest, and

could perhaps cause physiological alternations that contributes to the maintained decrease in maximal leg extension force.

The contraction speed may explain why the force decreased despite an small increase or no change in LLM, because rapid force production is dependent on the muscle activation and the speed-related properties of the muscle together with muscle size (16). The decreased leg press force could, therefore, be explained by a decline in the muscle properties important for rapid force production not investigated here, like rapid activation and muscle fiber type. Nindl et al (3) showed a full recovery of CMJ within five weeks, which may give an indication of the recovery time needed for explosive strength parameters.

Even if we could not detect any sex differences, it does seem like the observed decrease in maximal leg extension force is mainly explained by the changes in men, as there were no significant changes at any time point for the women. A decrease in physical performance for men and not for women after a strenuous FEX is not in accordance with previous studies of FEX (11, 12). A faster recovery in physical performance for women has, on the other side, been previously reported (12), which is consistent with our findings. Both of these observation may fit well with other reports indicating that women have a better ability to oxidize fat on submaximal intensities and to be more resistant to muscle fatigue than men (8, 13, 14, 29, 30). Considering that the relative reduction in FM during the FEX in our study did not display any sex difference, and that women generally have a higher amount of FM (22), this supports the indications of women having a more fat-predominant metabolism than men, which may explain the non-significant change for women in maximal leg extension force.

Changes in regulators of protein balance

For the sexes combined, we could not detect any significant changes at T2 or T3 in the protein synthesis regulators p70S6K and 4E-BP1, including the total protein levels, the phosphorylation status and the p/t. There was only a tendency for increase in total protein levels of 4E-BP1 at T3 for men and women combined.

These results indicate that at least some of the main regulators of muscle protein synthesis were unaltered by the strenuous FEX, and that the regulatory capacity for muscle protein synthesis may increase after one week of recovery. Previous studies have reported both unaltered and increased protein synthesis in the responses of a strenuous FEX (9, 11). These studies show decrements in body mass, but do not report if there were any loss of muscle mass. The measured increase in LLM in our study could indicate that there may have been a positive protein balance at some point, but not at the exact time we extracted the muscle sample the day after the end of FEX. However, the unaltered regulators of protein synthesis in *m. vastus lateralis* at T2 further supports the notion that there were only minor changes in LLM. Since total LBM was reduced, we cannot rule out if protein synthesis was down-regulated in muscles in other body parts. This emphasizes the fact that muscle biopsies are only a momentarily picture of the different myocellular processes. If we would have extracted muscle samples several times we could potentially have been able to elucidate the time course more precisely.

4E-BP1-protein levels were slightly increased for men at T2, which resulted in a tendency for sex difference. There was also tendency for reduced p70S6K-protein levels in men at T3, which resulted in a tendency for sex difference at T3. Even if there were no sex differences, these findings could indicate that men up-regulate their capacity for regulation of protein synthesis in response to a strenuous FEX in a greater manner than women, and that the opposite occurs for the sexes in the recovery phase.

After the FEX, we detected increases in $AMPK_{P/T}$ for the sexes combined. This indicates increased autophagy, which has been reported earlier after a strenuous FEX (9, 11). However, there were no change in Ulk1-protein levels or AMPK protein levels, and there were also no changes in the phosphorylation of $Ulk1^{Ser555}$ and $AMPK^{Thr172}$. Therefore, our results display only a minor acute effect on autophagy after a strenuous FEX for the sexes combined. The unchanged $Ulk1^{Ser555}$ may not be surprising, given that phosphorylation of Ulk1 has been suggested to be related to exercise intensity, where studies including low-intensity activity reports unchanged phosphorylation (31). On the other hand, Moberg et al. (11) reported similar results for AMPK protein levels, but opposite for Ulk1-protein levels and phosphorylation of $Ulk1^{Ser555}$ and $AMPK^{Thr172}$. It seems unlikely that the study by Moberg et al. (11) included a greater degree of high-

intensity than our study. The energy deficit in our study also appears to be larger, which theoretically should have increased the autophagy process. It could be that we might have missed a possible increase in the activity of Ulk1, because Ulk1 has many phosphorylation sites, and the critical role in the activation of Ulk1 during glucose starvation appears to be the phosphorylation of the AMPK-mediated $Ulk1^{Ser317}$ or $Ulk1^{Ser777}$ (19). In our study, however, we investigated only $Ulk1^{Ser555}$, which may not have a critical function. Moberg et al. (11) investigated several sites and found increased phosphorylation of both $Ulk1^{Ser317}$ and $Ulk1^{Ser555}$. This implies that any changes in $Ulk1^{Ser555}$ could potentially reflect changes in $Ulk1^{Ser317}$ in our study. We did not observe any changes in $Ulk1^{Ser555}$, but we cannot rule out the possibility that other AMPK-mediated phosphorylation sites, or the mTORC-mediated site, were activated. Because mTORC1 is able to disrupt the interaction between Ulk1 and AMPK (19), one possibility could be that the food consumed by the soldiers at the end of the FEX on day ten, potentially stimulated the mTORC pathway until the next morning when the muscle sample was extracted, and enough to inhibit the AMPK and consequently Ulk1 phosphorylation. We did not, however, see any changes in the protein synthesis regulators, which suggest minor activation of mTORC after the FEX.

Opposed to Moberg et al. (11), we detected a sex difference in Ulk1-protein levels at T2 as a result of a tendency for the women to decrease whereas men had a non-significant increase. Reduced capacity in autophagy initiation may be a response in women in order to reduce breakdown of contractile proteins. This could then explain why women respond with no significant reduction in maximal leg extension force. $AMPK_{P/T}$ was also only significant for the men, indicating a greater stimulation of autophagy in men.

After one week of recovery, we report increased phosphorylation of $AMPK^{Thr172}$ and increased $AMPK_{P/T}$. This indicates that the autophagy may have a delay in activation following strenuous FEX. The delayed response may be explained by the high degree of physical activity during the FEX, which may attenuate protein breakdown (32). This activity is likely to have been reduced in the recovery period, which allows for repair and replacement of damaged proteins caused by the strenuous FEX, which may have caused the observed increase in $AMPK_{P/T}$ and $AMPK^{Thr172}$. Considering that the LLM is unaffected after one week of rest, while there is still a decline in maximal leg extension force, the proteins degraded may be related to the muscle force-generating capacity. The

increases in phosphorylated AMPK ^{Thr172} and $AMPK_{P/T}$ were, however, not reflected by any changes in Ulk1. This may indicate that the cells are under conditions of moderate glucose limitation and sufficient amino acids. This condition would allow activation of AMPK to phosphorylate metabolic enzymes to promote amino acid utilization for energy production, but would not completely inhibit mTORC1, which in turn may prevent Ulk1 activation (19). It is therefore possible that the increase in $AMPK_{P/T}$ and $AMPK^{Thr172}$ were related to other activities, such as glycolysis, fatty acid oxidation or mitochondrial biogenesis, and not to autophagy. Another possibility is that the increased $AMPK_{P/T}$ and $AMPK^{Thr172}$ were related to protein degradation in the ubiquitin-proteasome pathway. We did not measure any downstream markers in this pathway, so we cannot exclude that this was the main action of AMPK.

The increase in phosphorylation of $AMPK^{Thr172}$ at T3 was significant for men, and not for women. The same was displayed for $AMPK_{P/T}$. This indicate a stronger activation of AMPK in men that could result in a stronger stimulation of autophagy, which might further explain a faster restoration of maximal force in women than in men.

Overall, our results indicate that there may be a delayed autophagy response to strenuous a FEX and that there may be a stronger stimulation of autophagy in men compared to women both after FEX and after one week of recovery. However, the lack of activation of Ulk1 indicates that the role of AMPK may have been to activate the ubiquitin-proteasome pathway.

Changes in mitochondrial enzymes

Markers for the three major pathways of aerobic metabolism were investigated, with changes in CS, COX-IV, and HADH as markers for the tricarboxylic acid cycle (TCA), oxidative phosphorylation, and beta-oxidation, respectively. At T2 we report unaltered CS and HADH, as well as reduced levels of COX-IV, for men and women combined. This indicates that a strenuous FEX causes some alterations to the aerobic metabolism and that this potentially affects the oxidative phosphorylation the most. Reduced oxidative phosphorylation is likely to decrease the maximal rate of oxidative ATP production (33), and may be why we observe increased $AMPK_{P/T}$ at both T2 and T3. However, increased activation of AMPK should in theory increase mitochondrial

biogenesis (18), which is opposite to what our results display. It could be that this is a compensatory effect in order to inhibit further degradation of mitochondrial enzymes. This could also support the suggestion that the main action of AMPK was to degrade proteins in the ubiquitin-proteasome pathway.

Both unaltered and reduced CS activity and unaltered HADH in *m. vastus lateralis* have been reported after FEX and other forms of prolonged low-intensity training (11, 34, 35). However, this might differ between muscles, as it has been reported increases in CS and HADH in the *m. triceps brachii* during eight weeks of skiing with backpack (36). Boushel et al. (35) showed that after 42 days of skiing, the oxidative phosphorylation was maintained during ADP-stimulated respiration measured *ex vivo* by high-resolution respirometry, even if the mitochondrial capacity was reduced. It was therefore suggested that the mitochondrial oxidative capacity is in excess of the oxygen delivered to the muscles, and that muscle mitochondria are able to reduce its content without having any impact on the maximal oxygen consumption (35). However, we did not measure mitochondrial content, and are therefore unable to say if the same mechanisms that Boushel et al. (35) suggests apply to our results.

After one week of recovery, the CS levels became significantly reduced, indicating a decreased TCA cycle capacity. This was not reflected by reductions in HADH levels, but there was a tendency for reduced COX-IV levels. The observation of increased $AMPK_{P/T}$ and $AMPK^{Thr172}$ after one week of rest is not consistent with decreased metabolic markers, suggesting that the main action of AMPK could be protein degradation and perhaps even mitophagy. Inconsistent with our findings, it was reported no change in CS activity three to four days after completing a 32-days ski journey across Greenland (34). Our results could perhaps indicate that the reduction in COX-IV levels at T2 initiated a feedback loop, which would then reduce the TCA cycle activity and consequently the CS levels.

Only the women significantly reduced their COX-IV levels at T2, and tended to reduce CS levels at T3. Even if we could not detect any significant sex difference, this indicates that women could potentially be more affected in their aerobic metabolism compared to men.

Changes in Na^+K^+-pump

We are the first to measure the responses of the Na^+K^+-pump after a strenuous FEX. Our results show that the β1-component was reduced for men and women combined after the FEX and stayed reduced for at least one week of recovery. Inadequate maintenance of Na^+ and K^+ gradients over the sarcolemma could result in impaired action potential propagation and thereby causing inexcitable muscle fibers (37). This could be one reason to why we observe a decreased maximal leg extension force. However, the Na^+K^+-capacity may be overexpressed (38) and impaired action potential would most likely limit a sustained maximum voluntary contraction (37), and therefore probably not affect the maximal leg extension force measured in this study. Furthermore, women did not show any significant changes in maximal leg extension force at any time point, while they at the same time were the only sex showing significant reduction in Na^+K^+β1-levels at both T2 and T3. The reason for this may be that we investigated the β-component, which is a subunit to the Na^+K^+-ATPase complex where the α-subunit bears all functional domains of the enzyme (38). On the other hand, increasing experimental evidence suggests that the β-subunit is an indispensable element for the structural and functional maturation of the enzyme as well as its intracellular transport to the plasma membrane (38). The reduction of Na^+K^+β1-levels from T1 to T2 and T3 may therefore suggest reduced capacity in the regulation of the membrane potential.

Women seemed to be more affected Na^+K^+β1-levels at T2 than men, as shown with a tendency for sex difference. The men, however, decreased their Na^+K^+β1-levels at T3, which suggest that men have a delayed response. Neither of the sexes was recovered one week after the FEX.

Limitations

Unfortunately, we did not have the possibility to perform familiarization to the physical tests. The soldiers were not used to the testing apparatus before the study, and we have no knowledge of how much each soldier was accustomed to strength exercise in general. However, if anything this should have led to a lower performance at T1 and therefore a even larger decline in performance than reported.

Because we investigate a limited number of samples, the two missing values for all proteins at T3 could have weakened our results. We did not sample muscle biopsies at T4, which makes us unable to describe any relationship between myocellular changes and the reduced force at after two weeks of recovery. To get a more detailed picture of autophagy we should have measured phosphorylation of $Ulk1^{Ser317}$, but we did not have access to this antibody. Furthermore, we could have included some downstream targets of Ulk1. To get a better picture of the total protein breakdown we should also have included markers for the ubiquitin-proteasome complex, but, unfortunately, this was not possible. It is further uncertain how well homogenized samples represent the membrane fraction of the proteins, and we did not measure any membrane-bound proteins, so the findings need therefore to be verified by investigation of fractionated samples.

There was no control of what the soldiers ate during the first three days of the FEX and our measurement of energy intake may therefore be underestimated.

Perspectives

This report is a part of a larger study investigating the effects of a strenuous FEX and the recovery for both women and men. This study offers some insight into the myocellular mechanisms behind long lasting reduced force, but the physiological and muscular mechanisms are still not clear. However, this study clearly indicates that there is a dissonance in relying on body mass and body composition in performance estimation, as the lean leg mass, at least the way it was measures in this study, was above baseline at all testing days. Our results, together with previous findings, indicate that factors related to maximal muscle power are affected in a greater manner, and recover slower, than factors related to maximal muscle force by a strenuous FEX. Future studies on FEX should therefore include investigation of muscle activation and speed-related properties of the muscle as well as a longer recovery period in order to determine when the physical capabilities are fully recovered.

Practical implications

The soldiers' physical performance was decreased and not recovered after two weeks of rest, which has been observed in other studies as well (5, 12). This reduction highlights

that having a high level of physical performance is necessary in order to be able to perform adequately after a period of strenuous military training. Our study does indicate that women are affected less than men to a strenuous FEX, but the absolute performance of the women is still lower compared to men. Both of these aspects are important to consider when planning and executing military training and operations. The recovery time in between strenuous military training and operations should, nonetheless, be longer than two weeks in order to recover the physical capabilities for both sexes.

Conclusion

A strenuous military field exercise resulted in similar reductions in maximal force-generating capacity in leg extensor muscles for men and women, and this was reduced for at least two weeks. The regulators of protein balance indicated increased catabolism and/or inhibited anabolism, which lasted for at least one week. There was a sex difference in the change in protein levels of Ulk1, but this was not related to any difference in reduction of force-generating capacity. The results indicate that there may be sex differences in the myocellular response to a military field exercise, but further investigations are needed to verify these finding and elucidate on the functional consequences.

Conflict of interest

The author declares no conflict of interest

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