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# The Effects of a 10-day Military Field Exercise on Body Composition, Physical Performance, and Muscle Cells in Men and Women

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## ABSTRACT

**Purpose:** To investigate the effects of a demanding military field exercise on physical performance, body composition and muscle cellular outcomes in men and women. **Methods:** Ten men (20.5±0.5 years) and 8 women (21.4 ± 1.4 years) completed a 10-day field exercise consisting of extensive physical activity with food and sleep restriction. Acquisition of body composition, physical performance, blood and muscle biopsies samples were done before and 1,7 and 14 days after the exercise. **Results:** There were no sex differences in the response to the exercise. Body mass was decreased with 5.6±1.8% and fat mass with 31±11% during the exercise. Both were still reduced after 14 days (2.5±2.3%, $p$<0.001 and 12.5±7.7%, $p$<0.001 respectively). Isometric leg strength did not change. Peak leg extension torque at 240 $°\cdot s^{-1}$ and counter movement jump height were reduced with 4.6±4.8% ($p$=0.012) and 6.7±6.2% ($p$<0.001) respectively and was still reduced after 14 days (4.3±4.2%, $p$ = 0.002, and 4.1±4.7%, $p$=0.030). No changes occurred in fiber CSA, fiber types, proteins involved in calcium handling or HSP70. During the exercise, αB-crystallin levels decreased by 14± 19% ($p$=0.024) in the cytosolic fraction and staining intensity on muscle sections tended to increase (17±25%, p=0.076). MuRF1 levels in the cytosolic fraction tended to decrease (19 ± 35%) and increased with 85±105% ($p$=0.003) in the cytoskeletal fraction 1 week after the exercise. **Conclusions:** The field exercise resulted in reduced body mass and physical performance in both sexes. The ability to produce force at high contraction velocities and explosive strength was more affected than isometric strength, but this was not related to any changes in fiber type composition, fiber area, $Ca^{2+}$ handling or fiber type specific muscle damage.



**Key Words:** MUSCLE STRENGTH, RECOVERY, MUSCLE DAMAGE, MUSCLE FIBRES, HEAT SHOCK PROTEINS, CALCIUM

## INTRODUCTION

Operational training and demanding military field exercises are a common and an important part of military education and training. Such exercises often involve very high activity levels and energy expenditure combined with low energy intake and lack of sleep (1, 2). These factors leads to energy deficits that have been reported to be as high as 5,000-6,000 kcal per day over a three to seven-day period (1, 3-5), further leading to large losses of body mass, body fat and muscle mass (2, 4, 6, 7). These exercises have also been shown to negatively affect different aspects of physical performance including aerobic power (3), anaerobic performance (8), maximal and explosive muscle strength (2, 6, 7, 9), and performance in military specific physical tasks (6). In addition, the recovery of physical performance from such exercises seems to be slow as we have recently reported that explosive strength was not recovered within two weeks (4, 10), and not within 6 weeks after more prolonged exercises (11).

Reductions in physical performance may have negative consequences for mission success, and therefore it is important to come up with countermeasures to prevent the reduction in physical performance and/or to aid recovery. However, in a recent review, O'Hara et al. concluded that documentation for effective countermeasures against the negative impact of demanding military training on performance is lacking in the literature (12). Some have tried to increase the protein intake during field exercises, without any beneficial effect (13, 14). Consequently, better knowledge on the physiological mechanisms behind the performance impairments will be important in order to design effective countermeasures against the negative effects of operational training and field exercises. These mechanisms remain unclear, and there are multiple factors that can contribute to the observed reductions in performance. Previous studies have speculated that the changes in body

composition and endocrine changes can explain the decline in physical performance (2, 3, 7). However, there are only small to no correlations between the changes in lean body mass, endocrine changes and changes in performance after demanding military field exercises (4, 7, 11, 15). In a previous study from our group, all changes in body composition and hormones were recovered within one week, even though physical performance was reduced for more than two weeks. Furthermore, despite men losing a substantial amount of muscle mass while women maintained their muscle mass during the exercise there were no sex differences in performance changes. This demonstrates a discrepancy between changes in body composition, including muscle mass, and physical performance (10). Therefore, the mechanisms behind the reduced performance is probably more related to changes in muscle quality rather than muscle mass. Muscle damage related to the high activity levels is a possible mechanism and reports of large increases in serum creatine kinase (CK) in both men and women after such exercises (4, 10) certainly indicate that some muscle damage exists. However, a clear relationship between individual changes in CK levels and changes in performance has not been established (10).

We have previously reported that explosive strength and maximal power development are more affected and recover slower than maximal isometric strength in both men and women (4, 10). Therefore, it can be speculated that the underlying mechanisms behind the decline in muscle performance involve structural damages or disturbances to energy systems that are important for explosive strength rather than maximal strength. Since type II fibers have higher maximal contraction velocity than type I fibers, one possible mechanism could be that military field exercises negatively affect type II fibers to a larger degree than type I fibers with for example larger muscle damage in type II fibers or with a fiber type shift towards slower fiber types. A typical response to muscle damage is increased expression and

accumulation of heat shock proteins (HSP) during the recovery period. These proteins have previously been reported to bind and accumulate to areas within the cell with mechanical damage in both sexes (16, 17). The levels of HSP accumulated in muscle fibers correlate with the decline in force generating capacity after exercise (18, 19). Thus, HSP can give an indication to what degree muscle damage exists, and which muscle fiber type is affected by a demanding military field exercise.

Another possible mechanism might be related to impaired calcium ($Ca^{2+}$) handling and dynamics in the muscle cells. For example, prolonged periods of high activity and endurance training have been reported to lead to a dissociation of the channel stabilizing protein calstabin1 from the $Ca^{2+}$ release channel, the ryanodine receptor (RyR1) (20, 21). This dissociation can result in leaky channels at rest, impaired $Ca^{2+}$ release and therefore impaired exercise capacity, muscle function and fatigue resistance (20). Furthermore, in male participants, six 30-s all-out cycling bouts has been reported to lead to an extensive fragmentation of RyR1 also causing them to become leaky at rest leading to increased resting cytosolic $Ca^{2+,}$ impaired $Ca^{2+}$ release and depressed muscle force, especially at lower stimulation frequencies (21). Unfortunately, no previous study has investigated fiber type specific changes or changes in $Ca^{2+}$ handling after demanding military field exercises.

The majority of previous studies investigating the effects of demanding short-term military field exercises have only included male participants. Previous studies have reported that men have a higher absolute energy expenditure and energy deficit compared to women during field exercises. However, when adjusted for body mass or fat free mass no sex

differences exists (5, 10, 22). It has also been reported that women oxidize more fat (5) and loses less muscle mass (5, 10) during such short-term field exercises compared to men. We have also observed that that the recovery of vertical jump performance after a short but extremely demanding military field exercise differed between men and women despite similar physical performance decrements during the exercise (10). Therefore, it might be speculated that the physiological mechanisms behind changes in performance during and in the recovery phase from such short-term exercises differ between the sexes. However, these have not been investigated.

Therefore, the main aim of the current study was to investigate how different aspects of physical performance are affected by and recovers from a demanding short-term military field exercise. A special focus in this study was to investigate the underlying physiological and cellular mechanisms behind the decrements in physical performance and investigate any sex differences in these changes.

The main hypotheses were that factors related to explosive strength and maximal power would be more affected by a demanding field exercise than maximal strength. These changes would be related to more pronounced impairments in type II fibers compared to type I fibers, and that changes in calcium handling proteins are involved in this etiology. The impairments in explosive strength and power together with the muscle cellular outcomes were expected to recover faster in women than men.

# METHODS

## Participants

Ten men (20.5 ± 0.5 years, 181 ± 6.7 cm and 76.7 ± 6.4 kg) and 8 women (21.4 ± 1.4 years, 166 ± 3.1 cm and 65.4 ± 12.3 kg) was recruited from second year military students at the Norwegian Defense Cyber Academy. In May each year these students participate in a ten day long mandatory demanding military field exercise called “Cyber Endurance”. Participation in the study was voluntary and all participants were free to withdraw at any time. All students performed the mandatory field exercise regardless of their participation in the research study, and participation in the study did not affect the content of the field exercise. The project was evaluated by the Norwegian Regional Committees for Medical and Health Research Ethics. All participants provided a written informed consent and the study was performed in compliance with the Declaration of Helsinki.

## Experimental design

Blood sampling, measurements of body composition and testing of physical performance were performed before (pre) and 24 hours (post 24h), one week (post 1 wk) and two weeks (post 2 wk) after the “Cyber Endurance” exercise (figure 1). In addition, muscle biopsies from *m. vastus lateralis* were collected approximately 3 weeks before the exercise (pre), at post 24h and at post 1 wk. To reduce the numbers of biopsies the participants had to undergo, no biopsy was taken at post 2 wk. On each test-day, blood samples were collected, and body composition measurements were performed in the morning before breakfast (between 06:00 and 08:00). The physical tests were performed 2–5 hours after breakfast. Biopsies were collected between breakfast and the physical tests. All participants performed their individual testing at the same time of day (± 1 hour) each test-day.

**Procedures and protocols**

***Cyber Endurance exercise.*** The Cyber Endurance exercise lasted for ten days and consisted of a high degree of various types of physical activity, sleep deprivation and low energy intake to create a realistic and demanding operational environment. The exercise involved soldiers performing physically and mentally challenging military tasks in an outdoor environment. The exercise encompassed a range of activities, including cyber-specific tasks, marching, patrolling, and physical combat conditioning training, which lasted for several hours. Additionally, the soldiers had to carry their personal combat equipment weighing approximately 20 kg throughout the entire exercise. The exercise was designed to gradually decrease the sleep, rest and food and gradually increase the physical and mental demands over the course of 10 days. During the three first days the soldiers ate as normal and slept in the military camp. During this phase they had physical training in the morning between 06:00 to 08:00 before they ate breakfast. This physical training consisted of intense combat simulation training and included exercises such as carrying ammo-boxes, carrying fellow soldiers, lifting and turning big tires, agility training, push-ups, burpees etc. The rest of the day they had a mix of classroom lessons and more physical demanding activities such as marching and patrolling. In these 3 days they slept between 00:00 and 05:30. The rest of the exercise they slept out in the field and operational activity lasted for a minimum of 18 hours each day. The soldiers slept approximately two to three hours each night. During this phase the soldiers performed cyber specific engineering tasks together with physical tasks, such as marching, patrolling, combat conditional training (as described above) lasting for several hours each day. Both men and women received field rations containing approximately 900 kcal•day$^{-1}$ and they had no access to outside food. Both the officers following the exercise and the soldiers confirmed that all field rations were eaten. During the exercise the students

were divided into mixed-sex teams so both men and women performed the same exercises. However, each team solved the challenges as they wanted, and we have no control on how each team distributed different tasks, for example distribution of the load carried. Accelerometers (ActiGraph wGT3X-BT, ActiGraph, Florida, USA), worn on the non-dominant wrist, were used to estimate the activity energy expenditure 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 • LBM (kg) + 370 (23). Total energy expenditure was the sum of activity energy expenditure and basal metabolic rate. In absolute values, the average energy expenditure per day during the exercise was significantly larger in men (5775 ± 558 kcal•day$^{-1}$) compared to women (4717 ± 644 kcal•day$^{-1}$, p = 0.002). However, normalized for body mass there were no sex differences (men: 75.5 ± 5.7 kcal•day$^{-1}$•kg$^{-1}$, women: 72.9 ± 6.8 kcal•day$^{-1}$•kg$^{-1}$, p = 0.396). Both sexes had similar energy expenditure from day to day, ranging from 5350 to 5990 kcal•day$^{-1}$ for men and from 4640 to 5011 kcal•day$^{-1}$ for women. With 900 kcal•day$^{-1}$ of energy intake the estimated energy deficit during the last 6 days were larger in men (4899 ± 574 kcal•day$^{-1}$) compared to women (3819 ± 684 kcal•day$^{-1}$, p = 002). Normalized for body mass this was 64.0 ± 5.8 kcal•day$^{-1}$•kg$^{-1}$ for men and 58.6 ± 5.3 kcal•day$^{-1}$•kg$^{-1}$ for women (p = 0.063). The scientific team had no influence on the content of the exercise that was controlled solely by the Norwegian Defense Cyber Academy.

***Body composition.*** Body composition and body mass were measured with bioelectrical impedance analysis (BIA) on an InBody 720 machine (Biospace Co., Seoul, Korea) according to the manufacturer's instructions. The BIA was administered after an overnight fast between 06:00 and 08:00. The students were instructed not to shower or be physical

active from they woke up until the test was completed and told to go to the toilet prior to the measurements. The participants also stood upright the last 5 minutes before the test. Participants performed all measurements in their underwear. Compared to DXA measurements the Inbody 720 has been shown too systematically underestimate fat mass by 3.6 ± 1.3 kg (4) and has been reported to have an average test–retest measurement error in percent body fat of 2.3 and 2.6 percentage points and an ICC of 0.97 and 0.98 in men and women respectively (24).

***Blood sampling***. Blood samples were collected in the morning (06:00-08:00) in a fasted state to investigate anabolic and catabolic hormones and markers of muscle damage. The students were instructed not to be physical active in the period between they woke up and the blood was collected. Whole blood was mixed before clotting in room temperature for 30 minutes and centrifuged at 1300 g for 10 minutes. Serum was transferred into tubes and stored frozen at -80 °C until analyses. Serum was analyzed for cortisol (analytic CV: 6.8 %), testosterone (7.0 %), CK (3.9 %), at Furst Medical Laboratory (Oslo, Norway) and IGF1 (6.0 %) at The Hormone Laboratory (Oslo, Norway).

**Muscle biopsy analyses**

Muscle biopsies were sampled from the mid-portion of the m. vastus lateralis under local anesthesia (Xylocainadrenalin, 10 mg/mL+5lg/mL; AstraZeneca PLC, London,UK), with a modified Bergstrom-technique. Samples intended for immunohistochemistry were aligned and covered in O.C.T embedding matrix (CellPath, Newtown, UK) and frozen in isopentane cooled in liquid nitrogen. Samples for Western blotting were gently washed in cold physiological saline and fat, connective tissue, and blood were discarded before they

were weighed and snap frozen in liquid nitrogen. All samples were transferred to storage at −80°C for subsequent analysis.

**Immunohistochemistry**

Eight $\mu$m thick cross sections were cut at −20°C using a microtome (CM3050; Leica Microsystems GmbH, Wetzlar, Germany) and mounted on microscope slides (Superfrost Plus; Thermo Fisher Scientific, Inc., Waltham, MA, USA). The sections were then air-dried and stored at −80 °C until further analyses.

After being thawed the muscle sections were blocked for 60 min with 1% BSA (A4503; Sigma-Aldrich Corp., St Louis, MO, USA) in a 0.05% PBS-T solution (524650; Calbiochem, EMD Biosciences, Inc., San Diego, CA, USA).

One slide of the muscle cross sections was incubated with antibodies against MyHCI (1:500; DSHB Hybridoma Product BA-D5, deposited by Schiaffino, S) and dystrophin (1:500; ab15277; Abcam Plc), one were incubated with antibodies against HSP70 (Cat#ADI-SPA-810, Enzo Life Sciences) and dystrophin and one with antibodies against alfa-B crystallin (Cat#ADI-SPA-222, Enzo Life Sciences) and dystrophin at 4 °C overnight followed by incubation with appropriate secondary antibodies (Alexa Fluor, A11005 or A11001, Invitrogen, Inc.) for 1 hour at room temperature. Between stages, sections were washed for 3 × 10 min in a 0.05% PBS-T solution with gentle agitation. Muscle sections were covered with a coverslip and embedded in ProLong Gold Antifade Reagent with DAPI (P36935, Invitrogen Molecular Probes, Eugene, OR, USA) and left to cure overnight at room temperature. Muscle sections were visualized using a high-resolution camera (DP72; Olympus Corp., Tokyo, Japan) mounted on a microscope (BX61; Olympus Corp.) with a

fluorescence light source (X-Cite 120PCQ; EXFO Photonic Solutions Inc., Mississauga, Ontario, Canada). To compare staining intensities between muscle sections camera and software settings were fixed when taking micrographs of the sections stained against the HSPs. HSP staining intensity was analyzed and related to each fiber type using ImageJ with Fiji image processing package (25).

Fiber type distribution and fiber cross-sectional area were identified and analyzed using TEMA software (CheckVision, Hadsund, Denmark). Fibers showing positive stain against BA-D5 was quantified as a type I fiber, while fibers not stained (BA-D5 negative) was quantified as a type II fiber. For representative immunohistochemistry pictures see Supplemental Figure 1 (Supplemental Digital Content).

**Immunoblotting**

For extraction of proteins about 50 mg muscle tissue was mechanically homogenized by rotor-stator in 1 ml homogenizing buffer (T-PER$^{TM}$, Tissue Protein Extraction Reagent, 78510, Thermo Scientific Rockford, IL, USA) with 20 µl HaltTM protease, 10 µl phosphatase inhibitor cocktail (1861281, Thermo Scientific) and 10 µl EDTA (1861274, Thermo Scientific, Rockford, IL, USA). Samples were subsequently mixed for 30 minutes at 4°C and later centrifuged at 10000G for ten minutes at 4°C. The supernatant was aliquoted to microcentrifuge tubes and stored at -80°C until further analyses.

Additionally, about 50 mg of muscle tissue was homogenized and fractionated into cytosol, membrane, nuclear and cytoskeletal fractions, using a commercial fractionation kit according to the manufacturer's procedures (ProteoExtract Subcellular Proteo Extraction Kit,

539790, Calbiochem; EMD Biosciences GmbH, Schwalbach, Germany). All cell fractions were aliquoted to microcentrifuge tubes and stored at -80°C until further analyses.

Total protein concentrations was measured with a commercial kit (BioRad DC protein micro plate assay, 5000113, 5000114, 5000115; Bio-Rad Laboratories, Inc., Hercules, CA, USA) and measured using a filter photometer (Expert 96; ASYS Hitech Cambridge, UK), and calculated using the provided software (Kim Version 5.45.0.1; Daniel Kittrich).

Samples were analyzed by Western blotting. Between 4 to 25 µg of denatured proteins (depending on sample) were separated by electrophoresis by 4-12% gradient gels (NativePAGE, BN1002BOX, Invitrogen, USA or NuPAGE, NP0321BOX, Invitrogen, USA) at 200 volts for 45-60 minutes in cold MES SDS running buffer (NuPAGE NP0002, Invitrogen). Separated proteins were then transferred to a PVDF membrane (162-0177, Bio-Rad, CA, USA) with XCell II Blot Module (Invitrogen, CA, USA) at 30 volts for 90 minutes in cold transfer buffer (NuPAGE, NP00061) and an antioxidant (NuPAGE, NP0005). After transfer the PVDF membranes were blocked in a 5 % skim milk solution (EMD Millipore Corporation, Billerica, MA, USA) in TBS-t (Tris Buffered Saline, 170-6435, Bio-Rad, CA, USA & 0.1% Tween® 20, P7949-100 ml, Sigma Aldrich) for two hours at room temperature. After blocking, the membranes were cut into smaller portions based on the molecular weight (weight marker; Protein Ladder PS 11, 310005, GeneOn, Germany) of the appropriate proteins, and incubated in primary antibodies according to Supplemental Table 1 (Supplemental Digital Content, Overview over the antibodies used for Western Blotting in the study) overnight at 4°C with gentle agitation 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 diluted in a 1 % skim milk solution in TBS-t. After incubation with secondary antibody, the membranes were washed. Membranes were then incubated for 5 minutes with Chemiluminescent Substrate SuperSignal WestDura® (Extended Duration Substrate, Thermo Scientific, 34076, Rockford, IL, USA). Chemiluminescence was measured using ChemiDoc ™ MP Imaging System and analyzed using Image Lab ™ Software (Bio- Rad Laboratories, Hercules, CA, USA).

SERCA1 and SERCA2 are exclusively expressed in muscle fiber type II and 1 respectively. To account for the large sample to sample variation in muscle fiber type (26) the individual SERCA1 and SERCA2 data at each time point the values were adjusted for the fiber type composition measured in the corresponding IHC piece using the formulas

SERCA1 = SERCA1 expression measured by Western blotting/100•percentage type II fibers

SERCA2 = SERCA2 expression measured by Western blotting /100•percentage type I fibers

**Physical performance test**

After a general warm up, explosive strength performance of the leg muscles was tested with a counter moment jump (CMJ). This was followed by testing of maximal isometric and isokinetic torque in the leg extension dynamometer.

***General warm up.*** The general warm-up consisted of 10 minutes cycling with a low and self-selected intensity. During the last 3 minutes the students performed three 30 seconds bouts at a higher intensity.

***Maximal isometric and isokinetic torque in leg extension.*** Maximal isometric torque (MVC) in leg extension and peak torque during a leg extension at 240 $°·s^{-1}$ were tested in a dynamometer (Humac Norm, CSMi Solutions, Stoughton, MA, United Sates). The participants were seated with a 90 ° hip angle and were stabilized in this position using chest, hip and thigh straps. The input axis of the dynamometer was aligned with the participants' knee joint and the ankle was strapped to a lever arm. The participants held their arms in front of their chest during all tests. First, the participants performed three maximal isometric leg extension against the lever arm with a 90 ° knee angle. The contractions lasted for 5 seconds, and 60 seconds rest was given between each attempt. The participants were instructed to perform the muscle action as forcefully and quickly as possible. The attempt with the highest maximal torque was chosen for the statistical analyses. After one minute of rest, three maximal isokinetic leg extensions from 90 ° knee angel to full extension were performed against the lever arm at an angular velocity of 240 $°·s^{-1}$. There was 60 seconds rest between each attempt. The attempt with the highest torque recorded during the contraction was chosen for statistical analyzes. Strong verbal encouragement was given to the participants at all attempts.

***Counter moment jump.*** The CMJ test was performed on a force-plate (HUR Labs, Tampere, Finland). The participants were instructed to stand on the platform with feet at shoulder-width. Following a countdown from the test-administrator, the participants then completed the jump. The jump was performed with a flexion of the knee and hip joint to approximately 90 ° in the knee joint, followed by a rapid countermovement and extension of the lower extremities. Hands were placed on their hips throughout the entire movement. Each participant was given 3 attempts, with a 30-second rest between each attempt. If the third

attempt was highest additional jumps were performed. The results from highest jump were used in the statistical analyses.

**Statistics**

All statistical analysis was done in IBM SPSS (IBM SPSS Statistics, version 25, IBM Corp., Armonk, NY, USA). A Mixed Design ANOVA with sex as between subject factor, and time as within subject factor, was used to investigate changes over time within sexes and possible interactions between time and sex. Where the sphericity assumption was not meet, the Greenhouse-Geisser procedure was used to correct the degrees of freedom. A significant interaction between time and sex was followed up with pairwise comparisons with Bonferroni adjustments to compare the mean between different time points for each sex separately. Furthermore, sex differences in absolute changes and calculated percent change from pre-values at each time point were then evaluated with pairwise comparisons with Bonferroni adjustments for multiple comparisons. If there was no interaction between time and sex pairwise comparisons between different time points where done for men and women combined. All reported p-values from these tests are Bonferroni adjusted. Correlations were investigated with Pearson's *r*. Missing values were replaced (interpolated) for participants that did not attend one of the post-tests due to injury, sickness or other logistical restrains. Values were calculated by adding the average percentage change for the whole group to the participants' values in the previous test. This was done for one woman at post 1 wk in all biopsy analyses. If a participant lost data at two timepoints or more for an outcome the data from this participant was excluded from all timepoints for this outcome. This was the case for one man at the analyses for HSPs and the muscle fiber CSA. Statistical significance was set at an alpha-level of 0.05. Values are presented as mean ± standard deviation (SD).

## RESULTS

### Body composition

There was a significant main effect of time for body mass (F(2.1,32.8) = 40.3, $p <$ 0.001), muscle mass (F(3.0,48.0) = 4.361, $p = 0.009$), and fat mass (F(1.8,29.4) = 124.5, $p <$ 0.001), but no significant time*sex interaction in body composition (body mass: (F(2.1,32.8) = 0.41, $p = 0.750$), muscle mass: (F(3.0,48.0) = 1.3, $p = 0.298$), fat mass: (F(1.8,29.4.0) = 0.42, $p = 0.741$).

For the sexes combined, body mass decreased from pre to post 24 hours with 5.6 ± 1.8 % (4.1 ± 1.6 kg, $p < 0.001$) and were still reduced by 2.5 ± 2.3 % (1.8 ± 1.6 kg, $p < 0.001$) two weeks after the exercise was finished (figure 2A, table 1).

Mainly all loss in body mass was explained by loss in fat mass, which was reduced by 31 ± 11 % (3.2 ± 0.9 kg, $p < 0.001$) for the sexes combined 24 hours after the exercise compared to pre. Two weeks after the exercise it was still decreased by 12.5 ± 7.7 % (1.4 ± 0.9 kg, $p < 0.001$) (figure 2C, table 1).

Only the men decreased muscle mass from pre to 24 hours after the exercise with 1.8 ± 1.4 % (0.7 ± 0.5 kg, $p = 0.026$), and muscle mass was back to pre-values one week after the exercise. There was no significant difference in percentage changes in muscle mass between men and women (figure 2E, table 1).

**Leg extension**

There was no main effect of time (F(3.0,48.0) = 0.489, $p = 0.692$) and no significant time*sex interaction (F(3.0,48.0) = 0.222, $p = 0.881$) in peak isometric leg extension torque as this did not change for neither sex during the study period (figure 2B, table1).

For peak leg extension torque at 240 $°\cdot s^{-1}$ there was a significant main effect of time (F(3.0,48.0) = 7.423, $p < 0.001$), but no significant time*sex interaction (F(3.0,48.0) = 0.384, $p = 0.765$, figure 2D, table 1).

For the sexes combined, peak leg extension torque at 240 $°\cdot s^{-1}$ was reduced 24 hours after the exercise (–4.6 ± 4.8 %, $p = 0.012$), one week (-5.0 ± 5.4 %, $p = 0.011$) and two weeks (-4.3 ± 4.2 %, $p = 0.002$) after the exercise compared to pre (figure 2D, table 1).

**CMJ**

There was a significant main effect of time (F(3.0,48.0) = 12.96, $p < 0.001$), but no significant time*sex interaction (F(3.0,48.0) = 0.287, $p = 0.287$) for CMJ.

For the sexes combined, CMJ height was reduced by 6.7 ± 6.2 % ($p < 0.001$) 24 hours after the exercise. It was still reduced by 7.8 ± 5.1 % ($p < 0.001$) and 4.1 ± 4.7 % ($p = 0.030$) 1 and two weeks after the exercise (figure 2F, table 1).

**Blood variables**

The changes in blood variables are displayed in table 2.

For testosterone, there was a significant effect of time ($F(3.0,48.0) = 22.5$, $p < 0.001$) and a significant time*sex interaction ($F(3.0,48.0) = 21.3$, $p < 0.001$). The testosterone levels in men decreased by 38 ± 26 % ($p < 0.001$) 24 hours after the exercise. One week after the exercise it had increased compared to pre-values by 25 ± 25 % ($p < 0.01$) but were back to pre-values after two weeks. Testosterone levels in women were as expected low and no changes occurred during the study period.

There was a significant effect of time ($F(3.0,48.0) = 68.7$, $p < 0.001$), but no time*sex interaction ($F(3.0,48.0) = 0.58$, $p = 0.981$) for IGF1. For both sexes combined IGF1 decreased during the exercise and was 50 ± 9 % ($p < 0.001$) lower 24 hours after the exercise compared to pre and was still reduced by 11 ± 10% ($p = 0.007$) after one week of recovery. After two weeks recovery it was back to baseline.

For cortisol, there was a significant effect of time ($F(3.0,48.0) = 4.1$, $p = 0.012$), but no time*sex interaction ($F(3.0,48.0) = 0.35$, $p = 0.787$). For the whole group, the pairwise comparisons only revealed a tendency to change from pre to two weeks after the exercise with a 12 ± 10% ($p = 0.085$) decrease. However, no changes occurred from pre to 24 hours after the exercise. Interestingly, the woman had higher cortisol values than men at every time point ($p < 0.01$).

For CK, there was a significant effect of time ($F(1.7,24.8) = 20.319$, $p < 0.001$), but no time*sex interaction ($F(1.7,24.8) = 1.11$, $p = 0.355$). For both sexes combined, CK increased by 271 ± 180 % from pre to 24 hours after the exercise ($p = 0.004$). After one-week recovery it was back to baseline.

**Muscle fiber CSA and proportions**

For muscle fiber CSA there was no main effect of time for neither muscle fiber type I ($F(2.0,28.0) = 1.407$, $p = 0.262$) nor muscle fiber type II ($F(2.0,28.0) = 1.183$, $p = 0.321$). There was no time*sex interaction for neither muscle fiber type I ($F(2.0,28.0) = 0.460$, $p = 0.636$) nor muscle fiber type II ($F(2.0,28.0) = 0.170$, $p = 0.844$) as no changes occurred in neither sex during the exercise or recovery period (figure 3A).

For type I muscle fiber proportion there was no main effect of time ($F(2.0,30.0) = 0.786$, $p = 0.465$) and no time*sex interaction ($F(2.0,30.0) = 0.617$, $p = 0.564$) as no changes occurred in neither sex during the exercise or recovery period (figure 3B).

**Calcium handling proteins**

There were no significant main effect of time for SERCA1 ($F(1.4,22.8) = 2.764$, $p = 0.098$) or SERCA2 ($F(2.0,32.0) = 0.065$, $p = 0.937$) and no time*sex interaction for SERCA1 $F(1.4,22.8) = 0.472$, $p = 0.628$) or SERCA2 ($F(2.0,32.0) = 0.211$, $p = 0.811$, figure 4A and figure 4B).

For RyR1 content in the membrane fraction there was no main effect of time ($F(2.0,32.0) = 1.781$, $p = 0.185$), but a significant time*sex interaction ($F(2.0,32.0) = 5.621$, $p = 0.008$). The women had an increased content of RyR1 in the membrane fraction one week after the exercise with 45 ± 93 % ($p = 0.021$) while no significant changes occurred in men (figure 4C).

No RyR1 band appeared at lower molecular weights and together with the lack of reduction in the full size RyR1 content, this indicates that no fragmentation of RyR1 occurred.

There were no main effect of time ($F(2.0,32.0) = 1.576$, $p = 0.222$) and no time*sex interaction ($F(2.0,32.0) = 2.157$, $p = 0.132$) for protein content of calstabin1 in the membrane fraction (figure 4D). There was also no main effect of time ($F(2.0,32.0) = 1.397$, $p = 0.262$) or a time*sex interaction ($F(2.0,32.0) = 0.046$, $p = 0.950$) for the ratio between the membrane protein content of calstabin1 and RyR1. None of these variables changed in neither men nor women during the study period (figure 4E).

**Heat shock proteins**

For αB-crystallin levels in the cytosolic fraction there was a significant main effect of time ($F(2.0,28.0) = 5.399$, $p = 0.010$), but no time*sex interaction ($F(2.0,28.0) = 0.72$, $p = 0.931$). For the sexes combined, αB-crystallin was decreased by 13.5 ± 18.9 % ($p = 0.024$) 24 hours after the exercise and were back to pre-values after one week of recovery (figure 5B).

Because of technical difficulties we were not able to perform αB-crystallin analyses in the cytoskeletal fraction.

There were no main effect of time ($F(2.0,30.0) = 0.470$, $p = 0.630$) and no time*sex interaction ($F(2.0,30.0) = 0.835$, $p = 0.444$) for HSP70 protein levels in the cytosolic fraction (figure 5D).

For HSP70 in the cytoskeletal fraction there were a tendency to a main effect of time ($F(1.36,18.56) = 3.973$, $p = 0.051$), and no time*sex interaction ($F(1.36,18.56) = 0.833$, $p = 0.405$, figure 5E). However, no significant changes in HSP70 during the study was found in the pairwise comparisons.

Fiber type specific analyses of HSPs on muscle cross sections were only possible to investigate in 5 of the men and 4 of the women. In these individuals there was no indication of different HSP responses between fiber types. Therefore, the HSP results are presented independent of fiber type to so we could include all participants also in the HSP analysis.

For αB-crystallin staining intensity in both fiber types combined there was a significant main effect of time ($F(2.0,28.0) = 10.032$, $p < 0.001$) but no significant time*sex interaction ($F(2.0,28.0) = 0.63$, $p = 0.939$)

The group combined tended to increase αB-crystallin staining intensity with 17 ± 25 % (p = 0.076) 24 hours after the exercise compared to pre. One week after the exercise the staining intensity showed no significant different compared to pre values (–9.4 ± 21.7 %, $p = 0.186$). However, from 24 hours to one week after the exercise there were a significant reduction in staining intensity (p = 0.002, figure 5A).

The increase in αB-crystallin staining intensity showed a tendency to a positive correlation with the percent change in peak leg extension torque at 240 $°\cdot s^{-1}$ ($r = 0.454$, $p = 0.077$)

For HSP70 there were no main effect of time ($F(2.0,30.0) = 0.199$, $p = 0.821$) and no time*sex interaction ($F(2.0,30.0) = 0.294$, $p = 0.747$) as HSP70 did not change in neither sex during the study period (figure 5C).

**MuRF1**

For MuRF1 protein levels in the cytosolic fraction there were a tendency to a main effect of time ($F(2,30) = 2.535$, $p = 0.096$), but no time*sex interaction ($F(1.3,19.5) = 0.057$, $p = 0.875$).

For both sexes combined there were no change from before the exercise to 24 hours after exercise, but it tended to decrease with 19 ± 35 % ($p = 0.072$) 1 week after the exercise compared to pre values (figure 6A).

For MuRF1 protein levels in the cytoskeletal fraction there were a significant main effect of time ($F(2.0,30.0) = 9.095$, $p < 0.001$), but no time*sex interaction ($F(2.0,30.0) = 1.220$, $p = 0.309$)

For both sexes combined there were no changes in MuRF1 protein levels in the cytoskeletal fraction from before the exercise to 24 hours after the exercise. However, one week after the exercise the levels had increased with 85 ± 105 % compared to pre ($p = 0.003$, figure 6B).

**DISCUSSION**

In line with our hypotheses, the 10-day demanding military field exercise had larger negative effects on the ability to produce force at high contraction velocities and explosive strength compared to isometric strength, as peak leg extension torque at 240 $°\cdot s^{-1}$ and CMJ

height were significantly impaired, whereas no impairment was observed in isometric MVC leg extension torque. Furthermore, the ability to produce force at high contraction velocities was not recovered in the 2-week recovery period. Contrary to our hypotheses these findings could not be explained by any changes in the investigated proteins involved in the $Ca^{2+}$ handling systems, changes in muscle fiber composition or fiber type specific changes in fiber area and indicators of muscular stress. However, there was increased content of MuRF1 in the cytoskeleton fraction 1 week after the exercise. There were no observed sex differences in changes in physical performance, nor in changes in the muscle cells. The military field exercise led to a similar reduction in body mass in both men and women and this reduction was mainly caused by reduction in fat mass. Nevertheless, a slight reduction in muscle mass was observed in the men. The changes in blood biomarkers followed the expected changes related to energy deficiency and demanding exercise and did not differ between sexes. All alternations in blood biomarkers recovered within one week.

The reductions in explosive strength in the legs are similar to some previous studies (1, 6, 9, 13) and smaller than others (4, 10) of comparable length (7-14 days). The lack of change in maximal strength measured as isometric torque in leg extension is also in agreement with some studies (27) but contradict other studies (4, 13). The main reason for the conflicting findings regarding physical performance in different studies is probably related to differences in the field exercises. Studies with large reductions in performance typically include higher activity levels (6000 to 7000 $kcal \cdot day^{-1}$), more demanding physical activity, lower energy intakes (approx. 550 $kcal \cdot day^{-1}$) and hence a more negative energy balance (4, 10) while studies with similar reduction have approximately the same energy consumption, activity levels and energy intake as the current study (1, 9, 13). Despite the modest stress

caused by the exercise and energy deficiency, the recovery from the impairments in explosive strength and ability to develop force at high contractions velocities was prolonged and not fully recovered after two weeks of recovery. Prolonged recovery has also been reported after the more demanding field exercises described above (4, 10). This indicates that even military field exercises with moderate acute reductions in performance can still lead to long lasting reductions in explosive strength and muscular power. Such long lasting impairments in muscle power-generating capacity should be considered when planning military training and operations. The similar changes between men and women in physical performance during the exercise were expected based on the limited amount of previous studies with (10). However, in contrast to our previous study where women recovered faster in CMJ height and maximal power than men (10), there were no sex differences in the recovery process of physical performance in the present study. The reason for the different findings is unclear but might be related to the much larger declines in performance in our previous study caused by a more extreme field exercise with a much higher energy expenditure (approx. 90 $kcal \cdot kg^{-1} \cdot day^{-1}$ vs approx. 75 $kcal \bullet day^{-1} \bullet kg^{-1}$) and lower energy intake (approx. 550 $kcal \cdot day^{-1}$ vs approx. 900 $kcal \cdot day^{-1}$).

An interesting finding from the current study is that the demanding field exercise had no negative effect on maximal isometric torque but led to a long-lasting decline in the ability to produce force at high contractions velocities and explosive strength. We measured the power related abilities with the CMJ but also with the peak leg extension torque at 240 $° \cdot s^{-1}$. This was because we wanted to measure power using a non-complex exercise involving the same muscles and movement as the isometric test to rule out that reductions in CMJ performance was mainly related to other factors, such as pain or technique. Our finding that

explosive strength and power related measures are more affected than other aspects of physical performance after military field exercises is also supported by previous studies (4, 10, 13, 27), and a recent meta-regression (28). However, the physiological mechanisms behind the prolonged impairments in specific aspects of muscle function after military field exercises are unclear.

It has been speculated that the observed changes in body composition and in anabolic and catabolic hormones could explain changes in physical performance (2, 3, 7, 29). This is not supported in our results as there were generally small to no correlations between the changes in muscle mass, endocrine changes, and changes in performance during the exercise and all analyzed hormones had recovered after one week of recovery when physical performance was still depressed. Furthermore, only men lost muscle mass during the exercise, whereas the changes in performance were similar in men and women. The lack of relationship between changes in body composition and performance has been demonstrated previously by our group, where large sex differences in muscle mass were not accompanied by any differences in in physical performance (10).

The reductions in body mass and fat mass after the field exercise in the current study were similar (9) or lower (10) than the reductions reported in field exercises of comparable duration. The differences between studies can be explained by variations in energy expenditure and energy intake. Studies with larger changes in body composition than our study have in general included more demanding field exercises with larger energy deficits (4, 7, 10). In the current study almost all change in body mass could be explained by changes in fat mass in both sexes. However, some of the changes in body mass might also be because of

dehydration and low muscle glycogen stores after the excises. To minimize the effect of dehydration, we did the post measurement 24 hours after the exercise under the same standardized conditions as on the other timepoints. The lack of change in muscle mass in the female soldiers is in accordance with a previous study from our research group (10) and further supports the notion that women have a high ability to preserve muscle mass during demanding military field exercises. The almost no change in muscle mass in men and the lack of sex differences in changes in muscle mass are not in accordance to most studies investigating the effects of demanding field exercises in men (2, 4, 7) and to previous studies comparing men and women (5, 10). A possible explanation for the different findings might be related to the content of the field exercises, for example the size of the negative energy balance. In our previous study, the activity level and energy expenditure were larger (approx. 90 $kcal \cdot kg^{-1} \cdot day^{-1}$ vs approx. 75 $kcal \bullet day^{-1} \bullet kg^{-1}$) and energy intake lower (approx. 550 $kcal \cdot day^{-1}$ vs approx. 900 $kcal \cdot day^{-1}$) leading to a much larger energy deficit than in the current study. Another possible explanation might be differences in initial fat mass of the participants as the male participants in our previous study had very low initial mass while we observed normal initial fat mass in the male participants in the current study.

The reduction in the anabolic hormones, testosterone and IGF-1, confirms that the soldiers were in a catabolic state, which is in good agreement with the changes in body mass and fat mass. The changes in anabolic hormones is similar to reports in studies of comparable length and with similar negative energy balance (13, 30, 31). In more challenging (e.g. higher activity levels) or exercises with longer duration and larger total energy deficit, even larger reduction has been reported (7, 10). The changes in anabolic hormones are believed to mainly be related to the negative energy balance, and to a lesser degree to the lack of sleep (29).

Since explosive strength and power related measures appear to be more affected than other aspects of performance, the decline in performance should partially be related to mechanisms that affect the ability to produce force at high contraction velocities more than isometric force. One possible mechanism might be changes in muscle fiber composition or a greater negative effect on the type II than type I fibers. No previous studies have investigated fiber type-specific changes after a military field exercise. However, studies investigating the effects of short term hypocaloric diets reports a larger muscle fiber atrophy in type II fibers than type I fibers in both rodents (32) and humans (33). One study in rats reported an overall fiber type shift from fast fibers towards slow fibers can last for up to two weeks (34). Furthermore, such hypocaloric diets have been reported to reduce the maximal rate of force development, slowing of the maximal relaxation rate and to increase losses of force at high compared to low stimulation frequencies. (34, 35) . However, in the current study there were no changes in fiber area in neither type I nor type II fibers and no changes in fiber type composition. Therefore, a larger atrophy in type II than type I fibers, and a fiber type shift towards a slower fiber type cannot explain the reduced explosive strength in the current study.

Hamarsland and colleagues (4) suggested that damage to the contractile apparatus in muscle fibers is an important mechanism for impairment of physical performance. The increase in CK levels after the exercise in our study indicates that some muscle damage was present. However, the increase in CK values after the exercise were moderate compared to previous studies (4, 10), and none of the soldiers had indications of rhabdomyolysis. There was also no correlation between changes in CK values and reduced performance. Interestingly, αB-crystallin levels in the cytosolic fraction were reduced 24 hours after the exercise together with a tendency to increased staining intensity in the muscle sections. The

biological role of αB-crystallin and other small HSP is to act as protein chaperones and interact with denaturated and unfolded proteins (18). Small HSPs have cytoprotective properties as they translocate and accumulate in the cytoskeletal/myofibrillar fraction after muscle damaging activity (16). Increased staining on muscle section has been shown to reflect an accumulation in myofibrillar structures (16, 18). Consequently, the decreased levels in the cytosolic fraction together with the tendency to increased staining indicate that some translocation also occurred after the field exercise in the current study. Both eccentric muscle contractions and other forms of strenuous muscle exercise have been reported to result to translocation of small HSPs from the cytosolic fraction to the cytoskeletal/myofibrillar fraction, and increased synthesis of these protein the following days (16, 18, 19). Increased staining for αB-crystallin and decreased levels in the cytosol in the current study therefore further support that the muscle cells experienced some stress and damage during the field exercise. However, the increased staining intensity for αB-crystallin was in most cases evenly distributed across the muscle fibers and the punctuated and granular staining which is considered a marker for myofibrillar disruption that occur after eccentric and heavy load strength training (18, 36) was not observed. Therefore, the increased αB-crystallin staining in the current study probably does not indicate large disruptions of the myofibrillar structure but some type of other cellular stress that could affect performance. The exact type of stress indicated and what structures are affected is unclear, but the staining pattern is similar to what has been reported after blood flow restricted exercise (19). Interestingly, acute aerobic non-damaging exercise (running) has been reported not to affect total expression of αB-crystallin 24 hours to 7 days after exercise, even though translocation were not measured (37). It should be noted that we took our biopsies after a 10 day long field exercise, and we do not have a clear overview over when in this period the possible muscle damaging work occurred or if the stress was evenly distributed during these 10 days. Therefore, we do not know how long

after the damaging activity the biopsy was taken even though the energy consumption was very similar from day to day indicating that the stress was quite evenly distributed. Since the staining of αB-crystallin appears to decline quite rapidly from 48 hours after muscle damaging eccentric exercise (16), this makes it challenging to compare our results to those of others.

A tendency for a moderate positive correlation was found between the percent change in staining intensity of αB-crystallin and peak torque during isokinetic contraction at 240°/s. This is not in accordance with a study reporting a large negative correlation between percentage fibers positive with small HSPs (HSP27) and change in force generation capacity after unaccustomed muscle damaging eccentric exercise (18). However, after this kind of exercise larger acute accumulation of small HSPs probably represents muscles with larger muscle damage and therefore correlates negatively with change in force generating capacity. The situation is quite different in the current study where the amount of damaging exercise probably was much less, and hence tolerable for the participants. Increased levels of different HSPs have actually been speculated to lead to increased protection of myofibrillar structures during repeated exposure to maximal eccentric exercise (16). Thus, it might be speculated that the cytoprotective role of αB-crystallin protected the subjects with higher accumulation in the muscle fiber better against muscle damage from the exercise, which consequently results in less reduction in force generating capacity.

We did not see any significant changes in the HSP70 neither measured by IHC nor in the cytosolic and cytoskeletal fraction measured by Western blotting. This is surprising because increased staining intensity of HSP70 in muscle cross section and increased levels in the cytosolic and the cytoskeleton fraction has been reported after various modalities

including aerobic non-damaging exercise, BFR exercise and muscle damaging eccentric exercise (16, 18, 19, 37). However, studies in well-trained rowers have shown that HSP70 expression is higher after high-intensity exercise compared to lower intensity. Additionally, low-intensity endurance training does not always lead to increased HSP70 expression (38). This might explain our results since the field exercise mostly had low intensity prolonged activity with some amounts of high intensity. Another explanation for the lack of changes in HSP70 levels can be related to the timing of the biopsies. We collected the muscle samples 24 hours and one week after the exercise. Because the exercise lasted for 10 days, we cannot rule out the possibility that high activity during the first days increased HSP70 levels that returned to pre-exercise values before the first post-biopsy. Depending on type of activity, the typical HSP70 response after acute sessions usually consist of none to small changes during the first hours, before a more substantial increase after approximately 24 hours and reach a peak after approximately 48 hours (18, 19), with quite large individual differences. However, to the best of our knowledge, no study has investigated the time course for the HSPs response after several days with high daily energy expenditure, such as a military field exercise.

Type II fibers have been reported to be more prone to muscle damage after eccentric work than type I fibers (16) and we aimed to investigate if the same could be seen after a military field exercise, and whether this could be a possible explanation for the larger reduction in explosive strength compared to maximal strength. However, even though this was measured in only 5 men and 4 women so caution is warranted, the tendency to increase in αB-crystallin staining and no changes in HSP70 staining was similar in type I and type II fibers suggesting that both fiber types experienced similar stress  This is in contrast to a predominantly increased αB-crystallin staining in type I fibers after BFR exercise (19), and a stronger staining of HSP27 in type II fibers after traditional heavy strength training (36).

We found a tendency to increased protein levels of MuRF1 24 hours after the exercise and a large increase 1 week after the exercise in the cytoskeletal fraction. This was accompanied with a tendency to a small reduction in the cytosolic fraction 1 week after the exercise. MuRF1 is a E3 ligase is specifically expressed in muscle (39) and among it's known targets are several myofibrillar proteins including troponin 1, myosin heavy chains, actin and myosin light chains (39). The specific increase in the cytoskeletal fraction do indicate MuRF1 binding to and ubiquitinating myofibrillar proteins and targeting them for destruction by the 26S proteasome in the week after the exercise. However, cautioned is warranted since we did not measure total ubiquitination or activity in any of the subunits of the 26S proteasome. Furthermore, as discussed, we did not find any reduction in muscle fiber CSA.

Several studies have reported acute changes in gene expression and protein levels of MuRF1 after both strength and endurance exercise (40-42), but only a few studies have investigated this after military field exercises or other similar challenges. A previous study investigating the effects of a similar military field exercise on the activation of the ubiquitin-proteasome system reported a very small reduction of MuRF1 protein levels in muscle homogenate but no changes in measures of proteasome activity 1-2 hours after the exercise (43). We found the biggest changes 1 week after the exercise so with the different timing of the biopsies it is difficult to compare to our study. However, as the homogenization procedure used by Moberg et al. (43) do not include cytoskeletal proteins in the supernatant the results are somewhat similar to our finding of a reduced amount in the cytosolic fraction. Ultra-endurance running has been reported to lead to an acute increase in the mRNA expression and protein levels of MuRF1 together with increased proteasome activity in muscle homogenates (44, 45). Furthermore, 21 days of energy deficit caused by an reduction in energy intake and increased energy expenditure also increased mRNA levels of MuRF1 (46).

These studies indicate that a negative energy balanced caused by a high activity levels combined with reduced energy intake can led to breakdown of muscle protein through increased levels of MuRF1 and activation of the ubiquitin-proteasome system. Timing wise the increased MuRF1 in the cytoskeletal fraction corresponds to the reduced physical performance and MuRF1 has been reported to be fiber-type dependent and is preferentially induced in type II fiber atrophy (47). This correspond well with the larger reduction of explosive strength compared to isometric strength in the current study. Therefore, it can be speculated that breakdown of myofibrillar proteins plays a role in the prolonged reduced explosive strength reported in the current study and this should be further investigated in future studies.

Another possible mechanism behind the reduced explosive strength might be related to impaired $Ca^{2+}$-handling and $Ca^{2+}$-dynamics in the muscle cells. Reduced $Ca^{2+}$-release due to changes in RyR1 content or function or alterations in SR $Ca^{2+}$-content due to changes in SERCA content and/or function could cause reductions in explosive strength. However, there were only small to no changes in the content of RyR1, SERCA1, SERCA2 and calstabin1, and no sign of any dissociation of calstabin1 from RyR1 or fragmentation of RyR1. When the channel stabilizing calstabin1 dissociates from RyR1, it can cause $Ca^{2+}$ to leak and impair muscle function and fatigue resistance (20). The lack of dissociation in the current study was unexpected since this dissociation has been reported after various exercise modalities including prolonged periods of high activity, marathon running and high intensity endurance training in both humans and rodents (20, 21, 48). However, to the best of our knowledge this is the first study investigating this dissociation of calstabin1 from RyR1 after a military field exercise consisting of high daily energy expenditure. It might be that the activity patterns, and periods of lighter engineering task were too modest to induce a dissociation. Although this

mechanism cannot be excluded for exercises with larger reductions in performance, (10) it is therefore unlikely to be the primary cause for the reduced performance observed in this study. The physical demands to enter the Norwegian Defense Cyber Academy is quite high and physical training is an important part of the soldier's education. Therefore, the soldiers participating in this study were quite well-trained and it might also be speculated that their training status prevented any dissociation as it has been shown that this dissociation and other modifications of RyR1 is less pronounced as training status increases (48). However, a dissociation has been reported in trained cyclists after a 3 hour cycling protocol at 70% of $VO_{2max}$ and after a marathon in athletes at a recreational level (20). Although a detailed registration of the soldiers' training habits was not collected, it is unlikely that their training status was too high to induce dissociation. Most previous studies investigating the dissociation of calstabin1 from RyR1 have used immunoprecipitation of the RyR1 complex (20, 21, 48). Unfortunately, that method was not possible to use in this study. Therefore, this was analyzed in the membrane fraction where RyR1 mainly can be found. With the assumption that the dissociated calstabin1 will end up in the cytosolic fraction, a dissociation of calstabin1 should lead to a reduced calstabin1 and/or reduced calstabin1/RyR1 ratio in the membrane fraction. However, this method has not been verified so there may be that the used method is not suitable for detecting this dissociation.

A fragmentation of the RyR1 complex has been reported to cause the channels to become leaky at rest leading to increased resting cytosolic $Ca^{2+}$, impaired $Ca^{2+}$ release and depressed muscle force (21). Therefore, a similar fragmentation after demanding military field exercises could be related to the reduced explosive strength and ability to develop high power. However, there were no sign of fragmentation of RyR1. RyR1 fragmentation has previously been reported after high intensity interval training consisting of 30 seconds all out

efforts (21, 49, 50), but not after more prolonged exercise such as a marathon (21). Thus, it appears that RyR1 fragmentation is more likely to occur after maximal intensity, short-term exercise, as HIIT, rather than prolonged low-intensity activity such as typical for the field exercise conducted in this study

**Limitations**

The fact that both sexes performed the field exercise together in mixed teams ensured that the physical demands of the exercise were as similar as possible between men and women. However, we do not know how each team distributed their load carriage and other tasks, so we cannot rule out the possibility that in some or all teams, men were chosen to carry the heaviest equipment and perform more physical strenuous tasks. To get a rough estimate of differences between the sexes in the energy expenditure during the field exercise we estimated it using wrist worn accelerometers. The energy expenditure was higher in men in absolute values, but no sex differences were discovered when normalized to body mass. These data suggest that men and women experienced similar relative levels of physical exertion during the exercise. However, the accelerometers do not account for the load carried by the soldiers so we still cannot rule out differences in relative loads.

The study has a quite low sample size that might impact our outcomes, especially when comparing sexes. This makes it difficult to draw conclusions regarding sex differences, especially for the muscle biopsy outcomes where the variation is generally larger. However, in most of our results, data from men and women are combined increasing the sample size and power.

As discussed earlier, we do not have a clear overview over when in the 10-day long field exercise the heaviest work was done and therefore do not have a complete overview of when compared to the hardest part of the exercise the post-tests are done.

## CONCLUSIONS

This is the first study to investigate the effects of a demanding military field exercise on muscle cellular outcomes and its role in explaining the prolonged impairments in physical performance after such exercises, and possible sex differences in this regard. The study confirms that explosive strength and the ability to generate force at high contraction velocities are the parameters that are most affected by demanding military field exercises and takes the longest time to recover in both sexes. Changes in these abilities were not recovered two weeks after the moderately demanding field exercise.

These reductions in performance were not related to the investigated changes in $Ca^{2+}$ handling, changes in muscle fiber composition or fiber type specific changes in fiber area, or muscular stress measured by translocation and increase in HSPs. However, the increased levels of MuRF1 in the cytoskeleton fraction one week after the exercise might be related to the reduced explosive strength. Furthermore, unlike our previous study, we did not observe any sex differences in body composition or physical performance during the exercise or recovery period, and the lack of sex differences may be explained by the less strenuous activity resulting in only modest alterations in both sexes.

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## FIGURE LEGENDS

**Figure 1**: Timeline over the study. The arrows represent when tests were performed. Three weeks before the cyber endurance exercise a resting muscle biopsi from m. vastus lateralis was collected. One week before the exercise, measurements of body composition and physical performance was done, and venous blood was collected. All these tests were repeated 24 hours (post 24h), 1 week (post 1 wk) and 2 weeks (post 2 wk) after the exercise, except that muscle biopsy was not collected at post 2wk.

**Figure 2:** Percent changes in body composition (left column) and physical performance (right column) from before (Pre) to 24 hours after the exercise (Post 24h) and during the first two weeks of recovery (post 1 wk and post 2 wk) for men (solid lines) and women (dotted lines). Thick lines with markers are the group mean, while thin lines represent individual changes. A: Body mass. B: Isometric peak torque (PT). C: Fat mass. D: PT at $240°•sec^{-1}$. E: Muscle mass. F: Counter movement jump (CMJ) height. Values are mean ± standard deviation. * Different than pre ($p < 0.05$) for men. # Different than pre ($p < 0.05$) for women. & Different than pre ($p < 0.05$) for men and women combined.

**Figure 3:** Changes in fiber area (A) and fiber type 1 proportion (B) from before (Pre) to 24 hours after the exercise (Post 24h) and during the first week of recovery (post 1 wk) for men (solid lines) and women (dotted lines). Thick lines with markers are the group mean, while thin lines represent individual changes.

**Figure 4:** Changes in heat shock proteins from before (Pre) to 24 hours after the exercise (Post 24h) and during the first week of recovery (post 1 wk) for men (solid lines) and women (dotted lines). A: αB-crystallin staining intensity measured by immunohistochemistry. B:

αB-crystallin content in the cytosol fraction. C: HSP70 staining intensity measured by immunohistochemistry. D: HSP70 content in the cytosol fraction. E: HSP70 content in the cytoskeleton fraction. # Tendency to different than pre for the men and women combined ($p < 0.1$).

**Figure 5:** Changes in protein related to calcium handling from before (Pre) to 24 hours after the exercise (Post 24h) and during the first week of recovery (post 1 wk) for men (solid lines) and women (dotted lines). A: SERCA1 content. B: SERCA2 content. C: RyR1 content in the membrane fraction. D: Calstabin1 content in the membrane fraction. E: Calstabin1/RyR1 ratio in the membrane fraction. * Different than pre ($p < 0.05$) for men or women

**Figure 6:** Changes in protein levels of MuRF1 from before (Pre) to 24 hours after the exercise (Post 24h) and during the first week of recovery (post 1 wk) for men (solid lines) and women (dotted lines). A: MuRF1 content in the cytosol fraction. B: MuRF1 content in the cytoskeleton fraction.

**SUPPLEMENTAL DIGITAL CONTENT**

**SDC 1:** Supplemental Digital Content.docx

Figure 1

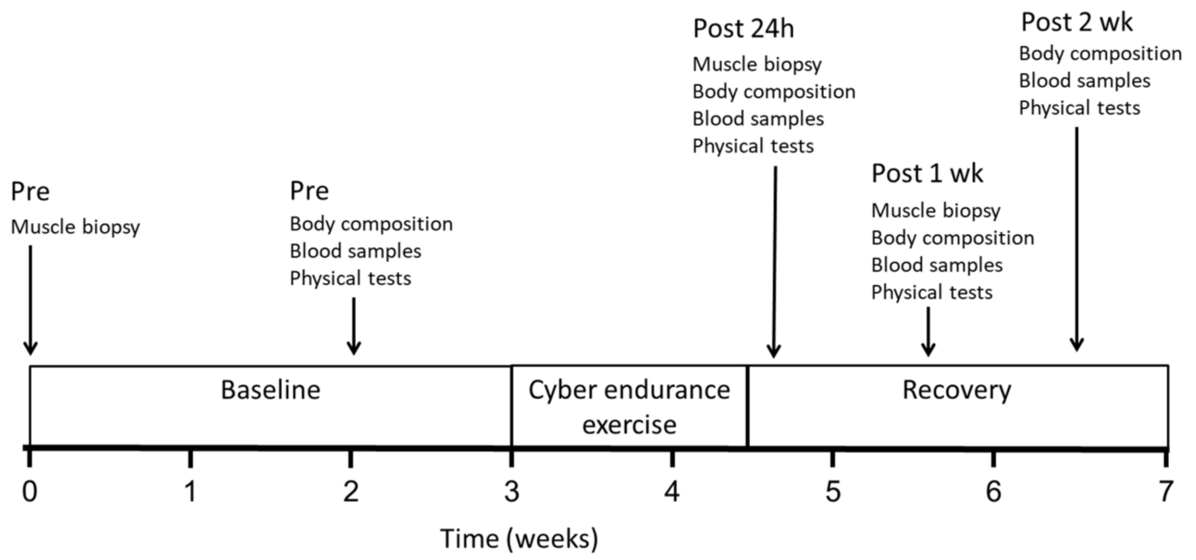

Figure 2

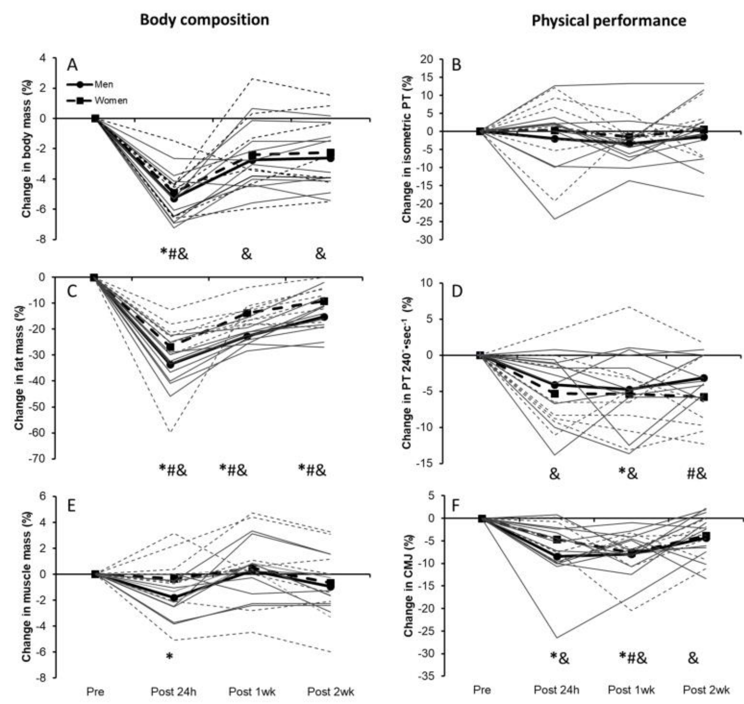

Figure 3

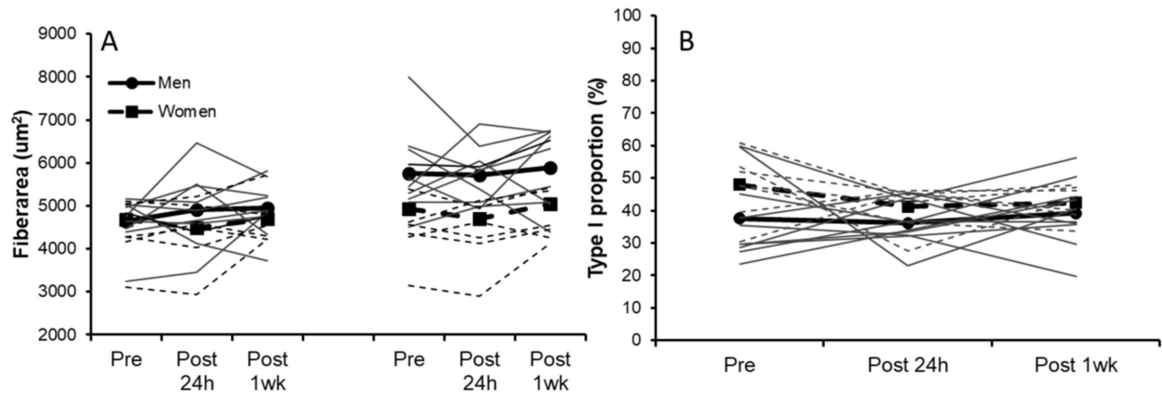

Figure 4

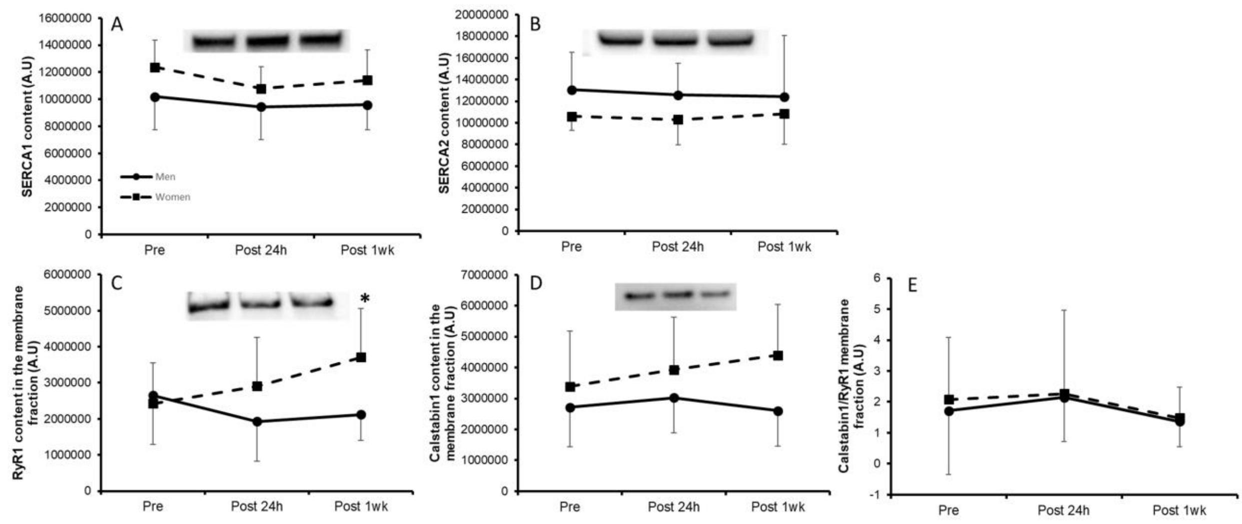

Figure 5

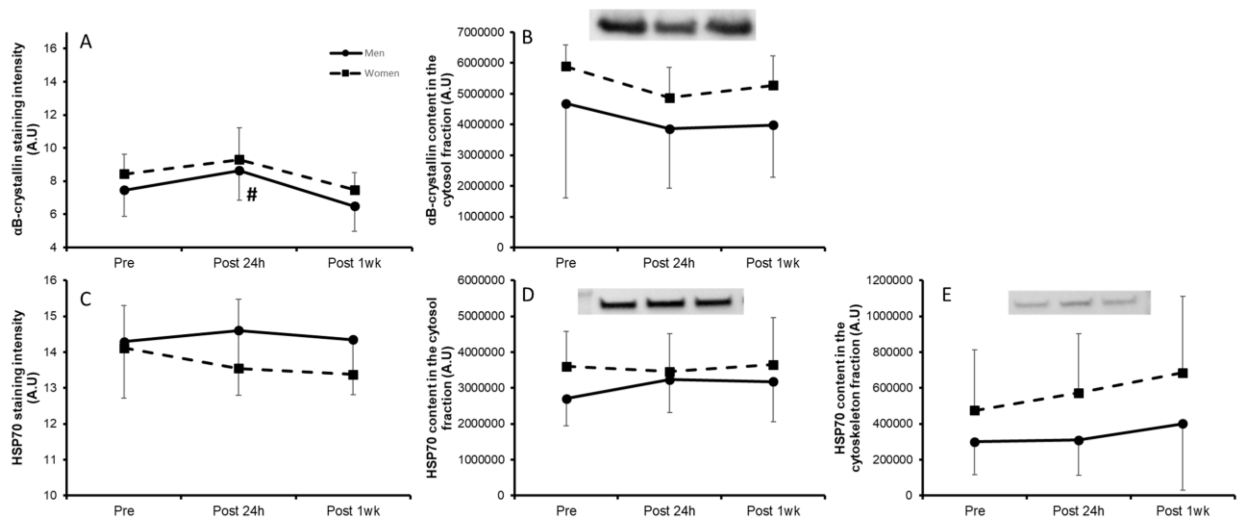

Figure 6

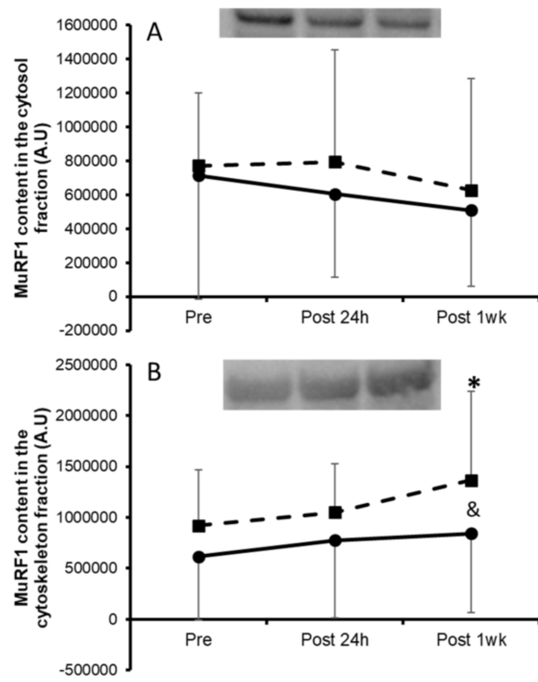

**Table 1. Body composition and physical performance in respond to the cyber endurance exercise and the following 2 weeks of recovery in both men and women**

| Variable | | Pre | Post 24 h | Post 1 wk | Post 2 wk |
|---|---|---|---|---|---|
| BM (kg) | Men | 76.7 ± 6.4 | 72.6 ± 6.3* | 74.5 ± 5.6 | 74.6 ± 5.8 |
| | Women | 65.4 ± 12.3 | 62.1 ± 11.1* | 63.6 ± 10.9 | 63.8 ± 11.4 |
| MM (kg) | Men | 38.2 ± 3.6 | 37.5 ± 3.4* | 38.3 ± 3.0 | 37.8 ± 3.2 |
| | Women | 28.8 ± 3.7 | 28.6 ± 3.3 | 28.8 ± 3.3 | 28.5 ± 3.4 |
| FM (kg) | Men | 10.0 ± 2.6 | 6.7 ± 2.1* | 7.7 ± 2.1* | 8.5 ± 2.3* |
| | Women | 14.1 ± 6.4 | 10.9 ± 5.9* | 12.2 ± 5.7* | 12.8 ± 6.0* |
| CMJ height (cm) | Men | 38.0 ± 8.6 | 35.1 ± 9.3* | 35.2 ± 8.9* | 36.5 ± 9.1 |
| | Women | 28.8 ± 4.2 | 27.4 ± 4.1 | 26.4 ± 3.8* | 27.6 ± 3.6 |
| Isometric PT (Nm) | Men | 258 ± 39 | 255 ± 53 | 250 ± 45 | 255 ± 49 |
| | Women | 188 ± 59 | 189 ± 69 | 186 ± 64 | 187 ± 53 |
| PT 240 °•$sec^{-1}$ (Nm) | Men | 121 ± 21 | 115 ± 21 | 115 ± 19* | 117 ± 20 |
| | Women | 79 ± 20 | 74 ± 19 | 74 ± 18 | 74 ± 16* |

Values are mean ± standard deviation. * Significantly different from pre. BM: Body mass, MM: muscle mass, FM: fat mass, CMJ: Counter movement jump, PT: Peak torque.

**Table 2. Serum levels of blood biomarkers in respond to the selection exercises and the following 2 weeks of recovery in both men and women**

| Variable | | Pre | Post 24 h | Post 1 wk | Post 2 wk |
|---|---|---|---|---|---|
| Testosterone (nmol·L$^{-1}$) | Men | 18.1 ± 3.5[a] | 10.9 ± 3.6[a]* | 22.4 ± 5.2[a]* | 19.7 ± 3.8[a] |
| | Women | 0.9 ± 0.2 | 0.8 ± 0.2 | 1.0 ± 0.3 | 0.9 ± 0.2 |
| Free testosterone index | Men | 6.2 ± 2.2 | 2.0 ± 0.6* | 5.6 ± 1.2 | 6.6 ± 2.2 |
| | Women | - | - | - | - |
| SHGB (nmol·L$^{-1}$) | Men | 31.6 ± 9.5 | 56.0 ± 13.1* | 40.8 ± 9.0* | 32.5 ± 10.5 |
| | Women | 98.6 ± 45.4 | 122.8 ± 46.0* | 110.2 ± 55.2 | 98.2 ± 46.6 |
| Cortisol (nmol·L$^{-1}$) | Men | 574 ± 101[a] | 569 ± 140[a] | 528 ± 98[a] | 481 ± 138[a] |
| | Women | 749 ± 110 | 805 ± 162 | 713 ± 128 | 682 ± 119 |
| Testosterone/Cortisol | Men | 3.3 ± 1.1[a] | 2.2 ± 1.4[a]* | 4.5 ± 1.5[a]* | 4.6* ± 2.4[a] |
| | Women | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 | 0.1 ± 0.0 |
| IGF-1 (nmol·L$^{-1}$) | Men | 29.3 ± 6.9 | 13.8 ± 2.1* | 25.5 ± 6.0* | 29.7 ± 7.2 |
| | Women | 29.3 ± 7.3 | 14.6 ± 2.8* | 26.2 ± 4.6 | 30.4 ± 10.2 |
| CK (U·L$^{-1}$) | Men | 208 ± 109 | 752 ± 497* | 175 ± 145 | 188 ± 100 |
| | Women | 292 ± 321 | 561 ± 269* | 123 ± 54 | 123 ± 54 |

Values are mean ± standard deviation. * Significantly different from pre, [a] significantly different from women at the same time point. SHGB: Sex hormone binding globulin, IGF-1: Insulin like growth factor 1, CK: Creatine kinase.

**Supplemental Table 1. Overview over the antibodies used for Western Blotting in the study**

| Antibody | Dilution | Catalog no | Lot no | Manufacturer | Sample | Load | Conc. (µg/ul) | Protein gel |
|---|---|---|---|---|---|---|---|---|
| SERCA1 | 1:2000 | MA3-930 | SK257991 | Thermo Scientific | Homogenate | 20 µl | 1 | NuPAGE™ 4 to 12%, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels (NP0321BOX, Invitrogen, USA) |
| SERCA2 | 1:500 | MA3-912 | SJ257196 | Thermo Scientific | Homogenate | 20 µl | 1 | |
| Calstabin1 | 1:1000 | ab2918 | GR3237577-1 | Abcam | Cytosol<br>Membrane | 20 µl<br>20 µl | 0.55-0.65<br>0.2-0.4 | |
| RyR1 | 1:8000 | ab2868 | GR3250452 | Abcam | Cytosol<br>Membrane | 30 µl<br>30 µl | 0.43<br>0.2 | NativePAGE™ 4-16% Bis-Tris Protein Gels, 1.0 mm, (BN1002BOX, Invitrogen, USA) |
| MuRF1 | 1:500 | Sc32920 | B2710 | Santa Cruz Biotechnology | Cytosol<br>Cytoskeleton | 20 µl<br>20 µl | 0.55<br>0.85 | NuPAGE™ 4 to 12%, Bis-Tris, 1.0–1.5 mm, Mini Protein Gels (NP0321BOX, Invitrogen, USA) |
| Secondary anti-mouse | 1:30000 | 31430 | IK1106742 | Thermo Scientific | | | | |
| Secondary anti-rabbit | 1:3000 | 7074s | 26 | Cell Signaling | | | | |
| Secondary anti-rabbit | 1:30000 | 31460 | TK274616 | Thermo Scientific | | | | |

RyR1: the ryanodine receptor, MuRF1: Muscle RING-finger protein-1

**Supplemental Figure 1:** Representative images from immunohistochemistry staining. A: αB crystallin staining. B: Fiber type and fiber CSA staining. Red is staining of dystrophin, green is staining of type 1 fibers, while non stained fibers are type 2 fibers.