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11 Neuromuscular performance and hormonal profile during military training and
12 subsequent recovery period
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49 Keywords: garrison training, military field training, maximal strength, endocrine, military
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ABSTRACT

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5 Introduction: Military training loads may induce different physiological responses in garrison and
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7 field training and only a little is known about how short-time recovery, lasting a few days, affects
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9 neuromuscular fitness and hormonal profile. This study aimed to investigate effects of garrison and
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11 field military service on neuromuscular performance and hormonal profile and to evaluate effects of
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13 a 3-day recovery on those factors.
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19 Methods: Twenty healthy male soldiers (20 ± 1 yrs.) participated in the study, which consisted of 4
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21 days of garrison training [days (D) 1-4] and 7 days of military field training (Days 5-12) followed
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23 by a 3-day recovery period (Day 15). Serum hormone concentrations [testosterone (TES), cortisol
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25 (COR), sex-hormone binding globulin (SHBG), free thyroxine (T4)] were assessed at D1, D5, D8-
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27 D12 and D15. Hand grip strength was measured in 10 participants at D1, D5, D8, D12 and D15.
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29 Maximal isometric force (MVC), EMG and rate of force development (RFD) of the knee extensors
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31 and arm flexors were also measured at D5, D12 and D15.
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39 Results: The maximal force of both the arm flexors and knee extensors were not affected by the
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41 garrison or field training, whereas the rate of force development (RFD) of the knee extensors was
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43 decreased during the field training (D5: 383 ± 130 vs. D12: 321 ± 120 N/s, $p < 0.05$). In addition,
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45 hand grip strength was mostly no affected, although a significant difference was observed between
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47 D8 and D12 (531 ± 53 vs. 507 ± 43 N, $p < 0.05$) during the field training. TES decreased already
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49 during the garrison training (D1: 18.2 ± 3.9 vs. D5: 16.2 ± 4.0 nmol/l, $p < 0.05$) and decreased further
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51 during the field training compared to baseline (D8: 10.2 ± 3.6 - D11: 11.4 ± 5.4 nmol/l, $p < 0.05$)
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53 exceeding the lowest concentration in the end of the field training (D12: 7.1 ± 4.1 nmol/l, $p < 0.05$).
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55 Similar changes were observed in free TES (D1: 72.2 ± 31.4 vs. D12: 35.1 ± 21.5 nmol/l, $p < 0.001$).
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1 The TES concentration recovered back to the baseline level and free TES increased after the
2 recovery period compared to the baseline values (D15: 19.9 ± 5.3 nmol/l, D15: 99.7 ± 41.1 nmol/l,
3 respectively). No changes were observed in the COR or SHBG concentrations during the garrison
4 period. COR was decreased in the end of the field training (D12: 388 ± 109 nmol/l) compared to
5 baseline (D1: 536 ± 113 nmol/l) ($p < 0.05-0.001$) but recovered back to the baseline levels after the
6 recovery period (D15: 495 ± 58 nmol/l), whereas SHBG linearly increased towards the end of the
7 field training ($p < 0.05-0.001$).
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19 Conclusions: The present findings demonstrate that neuromuscular performance can be relatively
20 well maintained during short-term garrison and field training even when a clear decrease in
21 hormonal profile is evident. In addition, hormonal responses during field training seems to be
22 greater compared to garrison training, however, the recovery of 3-day in free living conditions
23 seems to be sufficient for hormonal recovery. Therefore, a short-term recovery period lasting few
24 days after the military field training may be required to maintain operational readiness after the field
25 training.
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1 INTRODUCTION

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2 2 In military training, soldiers are exposed to physical and environmental stressors when preparing
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4 3 them to their demanding tasks. The challenge is to keep the balance between high physical loading
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6 4 combined with possible energy and sleep deficit, and sufficient recovery to maintain operational
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9 5 readiness. Although soldiers are intentionally pushed to their limits, recovery time and periods are
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11 6 needed to avoid overload^{1,2}. In order to improve military training, the monitoring of fitness and
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14 7 hormonal profile may play an important role for the management and programming of military
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16 8 education. From anecdotal evidence, it has been argued that there may be differences in training
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19 9 load between garrison and military field conditions. The field training, typically lasting several days
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21 10 or weeks, is considered physiologically generally the most demanding period in soldiers' military
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24 11 training partly due to energy and sleep deficit. Previous studies have demonstrated multistressor
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26 12 environment including high physical activity, energy deficit and sleep restriction in military field
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28 13 training^{3,4}. However, military training can contain high physiological and psychological stress
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31 14 sources also in garrison conditions^{5,6,7}. Nevertheless, there are not yet many studies that have
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33 15 addressed the differences in physical loading between the garrison and field conditions.
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38 17 Overtraining phenomenon is not widely used in military maybe due to its complex phenomenon and
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41 18 versatility of soldiers to overall stress sources. However, a severe overreaching and overtraining has
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43 19 previously been reported in soldiers. Chicharro et al. (1998) reported that 24 % of Spanish soldiers
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45 20 were in overtrained state after an 8-week basic training (BT) period⁸. Simultaneously, their physical
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48 21 performance (maximal isometric muscle strength, vertical jump and anaerobic capacity) decreased.
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50 22 In addition, Booth et al. (2006) concluded that 8 % of the studied Australian soldiers were in
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53 23 overtrained condition¹. These studies underline the importance of balancing between sufficient
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55 24 recovery and physical loading during military training. Furthermore, the previous studies
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58 25 collectively show negative changes in neuroendocrine function and fitness parameters during
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60 26 military field training^{8,9,10,11}. The previous studies have most consistently shown that neuromuscular
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27 function is decreased after arduous military field training when measured by vertical or horizontal
 28 jump test^{9,10,12,13}. Nevertheless, the mechanisms of neuromuscular function, such as EMG activity,
 29 has yet been rarely measured during the military field training in soldiers¹⁴.

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 31 In regard of recovery from physically challenging military training, one previous study showed that
 32 some components of physical fitness, however, may be recovered in young fit soldiers even during
 33 the military training⁹. Nevertheless, another study¹³ reported that a 4- day recovery is insufficient
 34 for muscular endurance to recover back to the baseline levels. Furthermore, hormonal recovery can
 35 be seen after one or several weeks following the field training^{3,15}. However, the information about
 36 shorter time-window for recovery has not been earlier assessed in soldiers. Despite the high
 37 physiological demands in soldiers during their training periods, to the best of our knowledge, there
 38 are no previous studies that have addressed the effect of recovery period lasting less than one week
 39 on physical fitness and hormonal profile parameters after military field training. Therefore, the
 40 present study aimed to investigate the effects of garrison and field services on neuromuscular
 41 physical fitness and endocrine profiles and further aimed to evaluate the effect of subsequent 3 days
 42 recovery. Lastly, this study adds to existing literature the novel assessment of EMG activity within
 43 military context during the military field training.

44 45 METHODS

46 47 Subjects

48 Twenty young (age 20 ± 1 yrs.) reconnaissance conscripts participated in the present study. The
 49 participants were healthy and fit men (mean \pm SD of 12-min running test: 2980 ± 267 m) who were
 50 informed of the experimental protocol and the measurements, and they gave their written consent to
 51 participate in the present study. They were also free to withdraw from the study at any time. The
 52 study protocol was approved by the National Defence Forces and the Ethics Committee of the local
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54 Study procedures

55 Training period consisted of 4 days of garrison training (days 1-4) and 7 days of military field
56 training (days 5-12) followed by a 3-day recovery period (days 13-15) (figure 1). The participants
57 had normal typical garrison training before the initiation of the study and, therefore, the baseline
58 values represent average physiological and physical conditions of the conscripts during garrison
59 training. Body composition variables were assessed at days 1 (D1), 5 (D5), 8 (D8), 12 (D12) and
60 day 15 (D15). Serum hormone concentrations [testosterone (TES), cortisol (COR), sex-hormone
61 binding globulin (SHBG), free thyroxine (T4)] were assessed at D1, D5, D8-D12 and D15. Hand
62 grip strength was measured in 10 participants at D1, D5, D8, D12 and D15. Maximal isometric
63 force (MVC), EMG and rate of force development (RFD) of the knee extensors and arm flexors
64 were also measured in 10 participants at D5, D12 and D15.

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66 The garrison training included lectures, military skill training and physical training as well as the
67 normal daily garrison routines such as preparing for the next training event, moving in formation
68 and having meals. The mean estimated energy expenditure was 3500 kcal (range 2100-4900 kcal)
69 based on heart rate, and the mean heart rate was 81 bpm/min during the garrison training. The
70 military field training included military skill training (3 days) which focused on rehearsing the basic
71 skills of a reconnaissance soldier followed by a 4 day rehearsal conducting reconnaissance during
72 offensive operations. The ambient temperature varied between + 4 ja – 10 degrees during the field
73 training and the mean estimated energy expenditure was 4100 kcal (range 1800-6400 kcal), and the
74 mean heart rate was 88 bpm/min during the field training.

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56 Body composition measurements

57 Body mass, fat mass and % body fat were measured in the morning using a bioelectrical impedance
58 method (InBody 720, Biospace Co., Ltd., Seoul, South Korea). Body height was measured by using
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79 a commercial scale.

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41 Physical fitness measurements and EMG recordings

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82 Maximal aerobic capacity was measured one week before the training period by 12-min running test
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93 to evaluate baseline level of the participants. The maximal running distance was counted in meters.

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84 Hand grip strength was measured with Jamar-Saehan dynamometer (Masan, Seoul, Korea)

separately from both hands. Result was calculated as a mean of best clenches of both hands.

Maximal strength of the upper and lower extremities was measured with isometric dynamometers

(University of Jyväskylä, Finland). The participants were instructed to produce maximal force as

fast as possible and to maintain it for 2 - 3 seconds during three maximal trials, separated by 2-3

min recovery periods, for the upper and lower extremities. The testing personnel encouraged

participants during the maximal effort. Knee extension and arm flexion were performed with the

right leg and arm, respectively, in a sitting position where knee and elbow angle was set to 90°. The

best performance was included for further analysis.

EMGs were recorded from the rectus femoris and biceps brachii muscles with bipolar surface

electrodes (Beckman miniature skin electrodes, 650437, Illinois, USA) according to the guidelines

of SENIAM¹⁶. The EMG signals amplification was 1000 times (Biotel 99, Glonner, Germany;

bandwidth 20-640 Hz / -3 dB; CMRR 110 dB), and it was digitized and synchronized with the force

records at a sampling frequency of 1 kHz. The EMG activities were full-wave rectified and

integrated. The maximal force and EMG values were calculated for the periods of 500 ms when the

force was kept on the maximal level. Maximal rate of force development (RFD) was defined as the

average force produced during the first 500 ms of the maximal force production.

Serum hormone concentrations

104 Blood samples were drawn from the antecubital vein in the mornings to analyze testosterone (TES),
105 cortisol (COR), sex-hormone binding globulin (SHBG) and free thyroxine (T4) (Immulite 1000,
106 Siemens Healthcare Diagnostics Products Ltd., Gwynedd, UK). Sensitivity of the measurements
107 were 0.5 nmol/l for TES, 5.5 nmol/l for COR, 0.2 nmol/l for SHBG and 3.9 pmol/l for T4. FreeTES
108 was calculated with Anderson formula: $\text{FreeTES (pmol/l)} = \text{TES (nmol/l)} \times \{2.28 - 1.38\} \times \log$
109 $[\text{SHBG (nmol/l)} \times 0.1] \times 10$ (Stenman 2000, 30).

110 Statistical analyses

111
112 Statistical analyses were done by using IBM Statistics software (SPSS 21.0.0). Mean values and
113 standard deviations (\pm SD) were calculated. Normality was tested with Shapiro-Wilk test. The
114 normally distributed data was analyzed by using repeated measures ANOVA with Bonferroni post
115 hoc for body composition, physical fitness and endocrine parameters. Friedman's test was used
116 when data was not normally distributed (T4, TES/SHBG-ratio and T4/COR-ratio). The level of
117 significance was set at $p < 0.05$.

120 RESULTS

121 *Body composition*

122 Body mass decreased during the field training (D8, D12) compared to the baseline values in the
123 garrison conditions (D1, D5) ($p < 0.001$), and did not fully increase back to the baseline level after
124 the recovery period (D1 vs. D15) ($p < 0.05$). Fat mass and body fat % decreased during the garrison
125 period (D1 vs. D5, $p < 0.001$) and were further decreased throughout the field training ($p < 0.001$).
126 Moreover, fat mass and body fat % remained at lower level after the recovery period compared to
127 the garrison conditions ($p < 0.05$). The detailed results of body mass and body composition are
128 shown in table 1.

130 *Neuromuscular performance*

131 The hand grip strength was mostly not affected by the training, although a significant difference was
132 observed between the days 8 and 12 during the field training ($p < 0.05$). Similarly, the maximal force
133 of both the arm flexors and knee extensors were not affected by the garrison or field training.
134 Maximal EMG of the biceps brachii muscle was higher in the end of the field training (D12) than
135 after the recovery period (D15) ($p < 0.05$), while maximal EMG of the rectus femoris muscle was not
136 affected. The rate of force development (RFD) of the knee extensors was decreased during the field
137 training (D5 vs. 12) ($p < 0.05$), whereas RFD of arm flexion was not affected (Table 2).

139 *Hormonal profile*

140 TES decreased already during the garrison training and decreased further during the field training,
141 exceeding the lowest concentration in the end of the field training ($p < 0.05-0.001$). However, TES
142 concentration was recovered back to the baseline level after the recovery period (Figure 2). Similar
143 changes were observed in free TES ($p < 0.05-0.001$), and after the recovery period, free TES was
144 increased to a higher level compared to its baseline value ($p < 0.001$) (Table 3). COR decreased in
145 the last days of the field training ($p < 0.05-0.001$) but recovered back to the baseline levels after the

146 recovery period (Figure 1). SHBG concentrations increased linearly towards the end of the field
147 training ($p < 0.05$ - 0.001), and it remained increased even after the recovery period compared to the
148 baseline values ($p < 0.001$) (Figure 1). The T4 concentrations were fluctuated during the whole two
149 week period, exceeding its highest concentration in the end of the field training ($p < 0.05$ - 0.001)
150 (Table 3). Moreover, no change in T4 between the baseline and after the recovery values was
151 observed (Table 3).

154 DISCUSSION

155 The main findings of the present study demonstrate that neuromuscular performance was not
156 negatively affected to a great extent during the military field training. Secondly, substantial changes
157 in hormonal profile during the military field training were observed. In addition, some of the
158 changes already existed after the garrison training, and the 3-day recovery period in free-living
159 conditions was sufficient to recover hormonal concentrations back to the baseline levels. Thirdly,
160 body fat decreased during the field training and remained at the lower level after the 3-day recovery
161 period.

162
163 Only small changes were noticed in the neuromuscular fitness during the study period. The
164 maximal force of the upper extremities, measured by grip strength was not altered, except a
165 difference between the day 8 and day 12, and similarly, isometric maximal arm flexor performance
166 as well as rate of force development did not change during the study period. On the contrary, EMG
167 of biceps brachii was increased after the field training, however, it decreased back to the baseline
168 level after the recovery period. The present results conclude that the neuromuscular performance of
169 the upper extremities can be well maintained, although great changes in the hormonal values were
170 observed at the same time. On the other hand, the increased EMG of biceps brachii without changes
171 in maximal force of the arm flexors after the field training may indicate minor decrement of the
172 excitation-contraction coupling process at muscle level. However, this has not caused any major
173 changes in the performance level of the neuromuscular system.. It may have seemed rational that
174 some decrements would be observed in the lower extremities, because of a high amount of running
175 and especially walking and standing typically occurs during the the field training. It seems that the
176 neuromuscular level of the conscripts in the present study was high enough to tolerate the high
177 physical activity during the field training. Previous studies have reported mixed results. A study by
178 Nindl et al. (2002) showed that military field training lasting 72 hours did not effect on bench press
179 power performance⁹, whereas a study by Knapik et al. (1990) reported that the 5-day field military

180 field training induced decreased upper body strength in most but not all upper body performance
181 tests¹⁷. In addition, they reported decrements in upper body muscular endurance (push-ups).
182 Similarly, a military field training lasting 21 days decreased upper body muscular endurance (push-
183 ups), which did not recover after 4-days¹³. Thus, it may be speculated that longer field training may
184 most potentially elicit greater changes in upper body performance than shorter ones. However,
185 obviously the military tasks performed during the field training is of importance and therefore
186 differences between studies are observed. The present study revealed that the level of
187 neuromuscular fitness of the study participants was at sufficient level to tolerate the high
188 psychological and physical demands set by the field training.

189
190 Although maximal knee extensor force and EMG of rectus femoris were unaffected during the
191 study period, a decrease in RFD was observed in knee extension. This finding extends the previous
192 studies, which have found either vertical or horizontal jump performance to decrease acutely after
193 military field training^{9,12,13} or during longer military training period lasting several weeks^{10,18}. From
194 physiological point of view, it may seem rational that some decrements are observed in the lower
195 extremities, because of a high amount of running and especially walking and standing typically
196 occurs during the military field training. The decrease in RFD together with previous findings with
197 decreased jump performance indicates a fatiguing phenomenon of the lower extremities, which may
198 originate from central and/or peripheral fatigue during the field training. The observed decrements
199 in lower body performance in the present study may also partly relate to load carriage, which has
200 been reported to decrease neuromuscular performance of lower extremities¹⁹ and increase in ground
201 reaction force²⁰.

202
203 Some changes in hormonal profile were detected even after the short garrison period evidenced for
204 example by the subtle decrease in testosterone. Nevertheless, greater changes in serum hormone
205 responses were observed in the field training and, especially, steep reduction was observed in the

206 end of the training. As hypothesized, the field training thus affected greater changes in hormonal
207 profile compared to the garrison training. Previous studies have shown, similar to the present study,
208 changes in hormonal profile during military field training^{3,4}. Previously, Alemany et al. (2008)
209 reported reductions in IGF-1 and body weight after the 8-day military field training including high
210 energy expenditure and sleep deficit²¹. Similarly, other studies have shown large reductions in
211 anabolic hormonal concentrations and body weight after several weeks of military field
212 training^{21,22,23,24,25}.

213
214 Testosterone is effecting as an anabolic signaling to, for example promoting muscle and bone mass.
215 In turn, in the presence of high demands to either physical or psychological stress or combined,
216 testosterone concentration has been shown to decrease often dramatically such as in the present
217 study. In military field training, energy and sleep deficit attenuates further testosterone
218 levels^{21,22,23,24}. The decreased circulating testosterone levels are most likely, at least partly, related
219 to higher testosterone uptake in the steroid receptors. The great reductions in TES with concomitant
220 increase in SHBG in the end phase of the field training clearly indicate a reduction of anabolic
221 potential of the conscripts. Moreover, SHBG is known to inhibit the function of testosterone and
222 therefore bioavailability of testosterone is affected by the SHBG concentration.

223
224 Cortisol is typically released in response to psychological and/or physical stress and when increased
225 it can be considered to represent a catabolic hormonal state as it mobilizes energy and inhibits
226 anabolic actions by other hormones such as testosterone. In the present study, cortisol did not
227 increase in concomitant with dramatic decrease in testosterone. It may speculative be discussed that
228 there can be changes in proportion of bound cortisol and free cortisol. Unfortunately, the free
229 cortisol was not measured in the present study. Nevertheless, as both forms of cortisol are
230 biologically active this could explain in part of the decreased values despite of high energy demands
231 experienced in the field training.

232

¹₂33 As thyroid hormones are responsible for metabolism regulation, a small increase in T4 in the
³₄4 present study might demonstrate a small negative energy balance at the first four days of military
⁵₆5 field training and adaptation, thereafter, to the end of the field training. However, no constant
⁷₈6 pattern of changes in T4 but rather fluctuation around the mean during the military training was
⁹₁₀7 observed. Together these results combined with a decrease in cortisol may speculatively be a sign of
¹¹₁₂8 lesser requirement of facilitation of metabolism being indicative that the dramatic changes in TES
¹³₁₄9 and SHBG would be to some extent more due to sleep and energy deficit than increased physical
¹⁵₁₆10 load.
¹⁷₁₈11
¹⁹₂₀12
²¹₂₂13
²³₂₄14
²⁵₂₆15
²⁷₂₈16
²⁹₃₀17
³¹₃₂18
³³₃₄19
³⁵₃₆20
³⁷₃₈21
³⁹₄₀22
⁴¹₄₂23
⁴³₄₄24
⁴⁵₄₆25
⁴⁷₄₈26
⁴⁹₅₀27
⁵¹₅₂28
⁵³₅₄29
⁵⁵₅₆30
⁵⁷₅₈31
⁵⁹₆₀32
⁶¹₆₂33
⁶³₆₄34
⁶⁵₆₆35
⁶⁷₆₈36
⁶⁹₇₀37
⁷¹₇₂38
⁷³₇₄39
⁷⁵₇₆40
⁷⁷₇₈41
⁷⁹₈₀42
⁸¹₈₂43
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⁸⁷₈₈46
⁸⁹₉₀47
⁹¹₉₂48
⁹³₉₄49
⁹⁵₉₆50
⁹⁷₉₈51
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¹⁰¹₁₀₂53
¹⁰³₁₀₄54
¹⁰⁵₁₀₆55
¹⁰⁷₁₀₈56
¹⁰⁹₁₁₀57
¹¹¹₁₁₂58
¹¹³₁₁₄59
¹¹⁵₁₁₆60
¹¹⁷₁₁₈61
¹¹⁹₁₂₀62
¹²¹₁₂₂63
¹²³₁₂₄64
¹²⁵₁₂₆65

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342 Figure 1. Timeline and measurements during the study period

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344 Figure 2. Hormone profiles during the study period

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346 Table 1. The body composition values during the study period.

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348 Table 2. The neuromuscular performance and muscle activity (EMG) values during the study period.

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350 Table 3. The concentrations of free testosterone and free thyroxine during the study period.

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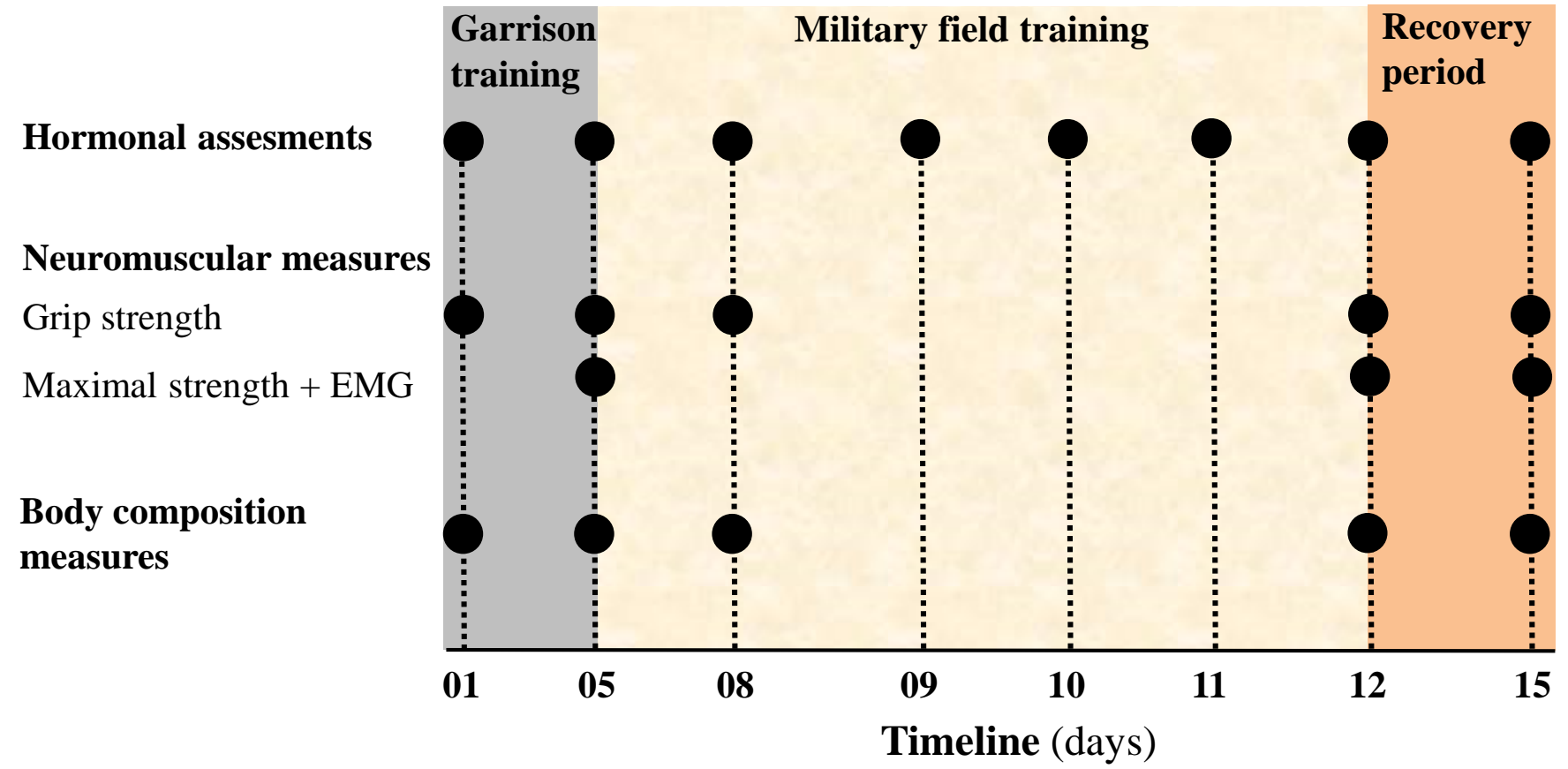
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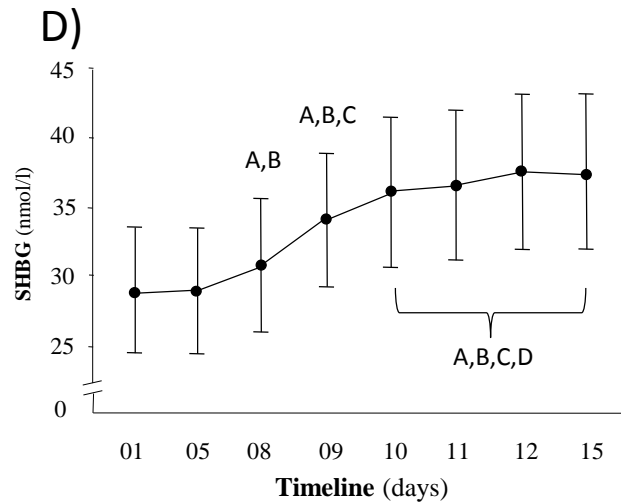
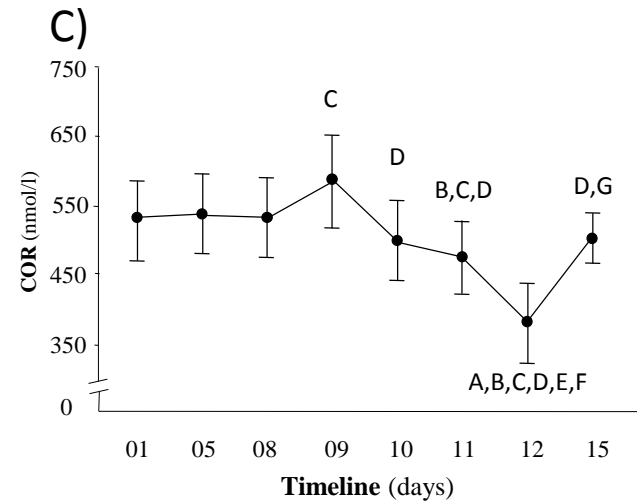
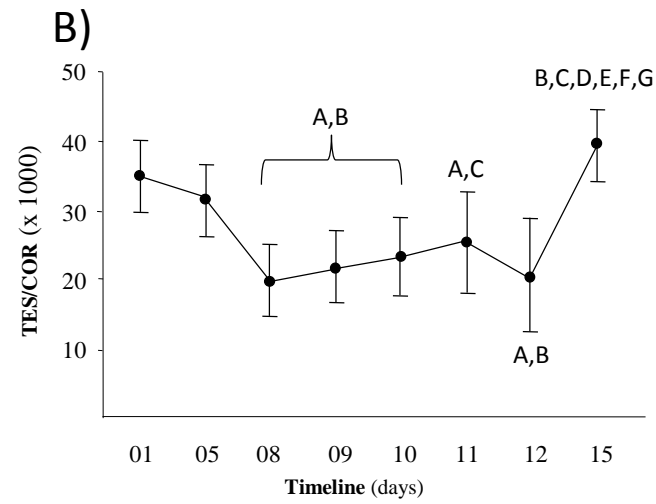
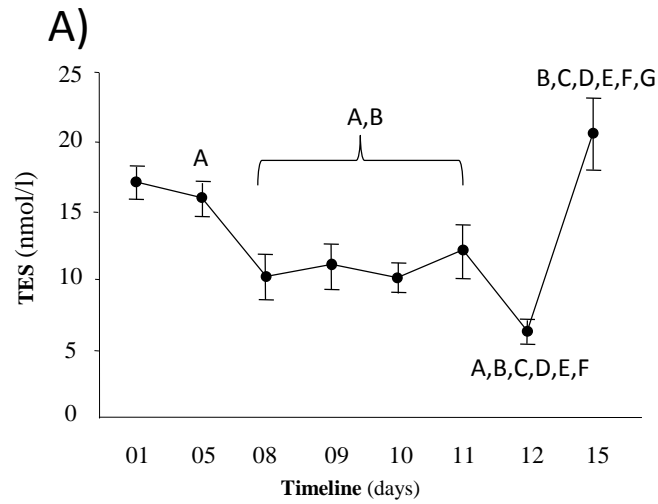
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A $p < 0.05$ compared to day 1, **B** $p < 0.05$ compared to day 5, **C** $p < 0.05$ compared to day 8, **D** $p < 0.05$ compared to day 9, **E** $p < 0.05$ compared to day 10, **F** $p < 0.05$ compared to day 11, **G** $p < 0.05$ compared to day 12

Table 1. The body composition values during the study period.

	D1 (garrison)	D5 (aftergarrison)	D8 (field training)	D12 (field training)	D15 (after recovery period)
Body mass (kg)	76.0± 7.1	75.7 ± 6.6	74.1± 6.7 aaa , bbb	73.1± 6.4 aaa , bbb , ccc	75.3± 7.1 a, c , ddd
Fat mass (kg)	11.2± 4.1	10.1± 4.1 aaa	9.0± 4.0 aaa , bbb	8.9± 3.8 aaa , bbb , ccc	9.2± 3.8 aaa, b , ccc
Fat%	14.5± 4.4	13.2± 4.5 aaa	11.9± 4.5 aaa , bbb	12.0± 4.5 aaa , bbb	12.1± 4.3 aaa, b

ap<0.05, **aaa** p<0.001 compared to D1, **b**p<0.05, **bbbp**<0.001 compared to D5, **c**p<0.05**cccp**<0.001 compared to D8,**ddd**p<0.001 compared to D12.

Table 2. The neuromuscular performance and muscle activity (EMG) values during the study period.

	D1 (garrison)	D5 (aftergarrison)	D8 (field training)	D12 (field training)	D15 (after recovery period)
Handgrip strength (N)	502± 77	526± 49	531± 53	507±43 c	502± 54
MVC of knee extension (N)	-	670± 219	-	590± 180	603± 240
MVC of arm flexors (N)	-	0.343± 0.051	-	0.354± 0.052	0.351± 0.050
RFD on knee extension (N/s)	-	383 ± 130	-	321 ±120 b	328 ± 120
RFD on arm flexion (N/s)	-	265 ±51	-	242 ±56	244 ±53
EMG of rectus femoris (μV)	-	0.169 ± 0.055	-	0.161 ± 0.045	0.167 ± 0.057
EMG of biceps brachii (μV)	-	567 ±231	-	647 ± 330	565 ±332 d

b=p < 0.05 compared to D5, **c**=p < 0.05 compared to D8, **d**=p < 0.05 compared to D12,

Table 3. The concentrations of free testosterone and free thyroxine during the study period.

	D1 (garrison)	D5 (after garrison)	D8 (field training)	D9 (field training)	D10 (field training)	D11 (field training)	D12 (field training)	D15 (afterrecov eryperiod)
FreeTES (pmol/l)	72.2 ±31.4	65.7±31.2	43.5±20.5a aa,bbb	53.0±28.9a aa, b, c	50.5±24.2a aa, b, c	55.6±30.8a aa	35.1±21.5aaa, bbb,ddd,eee,ff ff	99.7±41.1 aaa,bbb,c,d d,eee,fff,gg g
T4 (pmol/l)	15.8±2.1	16.5±1.7aa a	15.6±2.2 b	15.4±1.9bb b	15.4±2.0bb b	16.2±2.3c,d ,ee	17.1±1.9 a,b,ccc,ddd,ee e,f	15.4±2.0b,g gg

a/aaa=p < 0.05/0.001 compared to D1, b/bbb=p < 0.05/0.001 compared to D5, c/cc=p < 0.05/0.001 compared to D8, d/ddd=p < 0.05/0.001 compared to D9, e/eee=p < 0.05/0.001 compared to D10, fff=p < 0.001 compared to D11, ggg=p < 0.001 compared to D12.