Positive adaptation of HPA axis function in women during 44 weeks of infantry-based military training

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29

Abstract

Background

Basic military training (BMT) is a useful model of prolonged exposure to multiple stressors. 8–12 week BMT is associated with perturbations in the hypothalamic-pituitary-adrenal (HPA) axis which could predispose recruits to injury and psychological strain. However, characterisations of HPA axis adaptations during BMT have not been comprehensive and most studies included few if any women.

Methods

We studied women undertaking an arduous, 44-week BMT programme in the UK. Anxiety, depression and resilience questionnaires, average hair cortisol concentration (HCC), morning and evening saliva cortisol and morning plasma cortisol were assessed at regular intervals throughout. A 1-h dynamic cortisol response to 1μg adrenocorticotrophic hormone-1-24 was performed during weeks 1 and 29.

Results

Fifty-three women (aged 24 ±2.5 years) completed the study. Questionnaires demonstrated increased depression and reduced resilience during training (F 6.93 and F 7.24, respectively, both $p<0.001$) . HCC increased from 3 months before training to the final 3 months of training (median (IQR) 9.63 (5.38, 16.26) versus 11.56 (6.2, 22.45) pg/mg, $p=0.003$). Morning saliva cortisol increased during the first 7 weeks of training (0.44 ±0.23 versus 0.59 ±0.24 μg/dl $p<0.001$) and decreased thereafter, with no difference between the first and final weeks (0.44 ±0.23 versus 0.38 ±0.21 μg/dl, $p=0.2$). Evening saliva cortisol did not change. Fasting cortisol decreased during training (beginning, mid and end-training concentrations: 701 ±134, 671 ±158 and 561 ±177 nmol/l, respectively, $p<0.001$). Afternoon basal cortisol increased during training while there was a trend towards increased peak stimulated cortisol (177 ±92 versus 259 ± 13 nmol/l, $p=0.003$, and 589 ±164 versus 656 ±135, $p=0.058$, respectively).

55

56 **Discussion**

57 These results suggest a normal stress response in early training was followed quickly by habituation,
58 despite psychological and physical stress evidenced by questionnaire scores and HCC, respectively.
59 There was no evidence of HPA axis maladaptation. These observations are reassuring for women
60 undertaking arduous employment.

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1. Introduction

Stress can be defined as the response of an individual to a threat or challenge (a stressor) to maintain mental or physical allostasis (Selye, 1946). Basic military training is an ideal setting for the study of stress, since it entails prolonged exposure to multifaceted stressors, such as long days of physical work, restricted food intake and sleep, austere environments, time pressure and increasing responsibility while under continuous assessment by military instructors. Field exercises, a core component of basic military training, combine strenuous exertion over days or weeks with challenging scenarios of increasing complexity, in an unfamiliar, multi-stressor environment. The overall aims of basic military training are to test leadership and multi-tasking and develop traits like self-awareness and physical and mental robustness.

Cortisol, the main effector hormone of the hypothalamic-pituitary-adrenal (HPA) axis, is an important biological marker of stress. Cortisol is released in a pulsatile manner. Fasted morning plasma cortisol concentrations can be considered a 'stress' response to fasting or venepuncture (Reynolds et al., 2001b), whereas early morning salivary concentrations may provide information about the HPA and neurophysiological response to waking (Chida and Steptoe, 2009). Cortisol concentrations measured in urine or hair give additional information about activation of the HPA axis over longer durations (hours or months, respectively). Morning and evening sampling on the same day allows the diurnal cortisol slope to be calculated, with slope size inversely associated with a wide range of mental and physical health outcomes (Adam et al., 2017). Cortisol can also be measured in response to physiological stimuli (e.g. adrenocorticotrophic hormone, ACTH) to observe isolated HPA axis function, the size of response being associated with traumatic stress exposure (Golier et al., 2014), and increased risk of cardiovascular disease (Reynolds et al., 2001a) and reproductive dysfunction (Ackerman et al., 2013).

Sustained elevations in serum cortisol have been reported following stressful military captivity training (Taylor et al., 2007). Low concentrations of hair and saliva cortisol in response to social stress predict

subsequent development of post-traumatic stress disorder during military deployments (Steudte-Schmiedgen et al., 2015). Variations in cortisol concentrations have complex and multidimensional associations with a variety of biological and psychological disorders. For example, sleep deprivation is associated with relatively low wakening cortisol compared with the following evening (Abell et al., 2016a), while hair cortisol is positively associated with symptoms of depression (Abell et al., 2016b) and stress (Stalder et al., 2017), and negatively with anxiety disorders (Stalder et al., 2017). Higher average overnight serum cortisol is found in anorexia nervosa and functional hypothalamic amenorrhoea (Gordon et al., 2017). Overnight cortisol concentration is associated with lower bone mineral density (Lawson et al., 2009) and reduced gonadotrophin secretion (Ackerman et al., 2013) in women. Increased cardiovascular risk is associated with lower cortisol response to waking, higher average hair cortisol (Kuehl et al., 2015), and lower morning cortisol concentration compared with evening concentration (Kumari et al., 2011).

Previous studies of cortisol responses to basic military training have only been undertaken during short duration training. There was no effect of 10 weeks basic military training on hair cortisol concentration in male Swiss Army cadets (Boesch et al., 2015), while others have identified increased cortisol in 12-hour urine samples after 4 weeks military training among male Greek recruits (Makras et al., 2005) and in fasting blood samples after 9 weeks of basic military training among Australian Army male and female recruits (Drain et al., 2017). Conversely, Clow et al. (2006) demonstrated a reduction in the cortisol response to waking after 11 weeks of British basic military training in men and women. Some of the discrepancies between studies may be explained by differences in volume and intensity of exercise, a major component of basic military training; both are associated with acutely elevated cortisol concentrations (Skoluda et al., 2015; Zschucke et al., 2015). High intensity interval training during Australian basic military training has been associated with additional plasma cortisol elevations compared with extant, endurance-based training (Drain et al., 2017). Exercise is also associated with elevated hair cortisol concentrations (Skoluda et al., 2012), however overtraining syndromes, which

may occur in basic military training (Booth et al., 2006), may be associated with blunted dynamic cortisol responses (Cadegiani and Kater, 2017). Cortisol response to ACTH and/ or corticotrophin releasing hormone (CRH) may also be reduced by sleep deprivation, a common component of military training (Guyon et al., 2014). Whether long durations of military training are associated with a transient adaptation in the HPA axis, or if stress, and other factors, are associated with reduced dynamic function consistent with overtraining is unknown. Disruption of cortisol secretion may indirectly be related to risk of training-related injury from uncoupling of bone turnover, and in the long-term, reproductive function and mental health problems (Abell et al., 2016b; Ackerman et al., 2013; Gordon et al., 2017). We studied women since women in the military could be at greater risk of reproductive dysfunction (Gifford et al. 2017), are exposed to greater physiological strain (O'Leary et al., 2018), and are at a greater risk of training related injury (Blacker et al. 2008) and stress fracture (Wentz et al. 2011) than men.

This study aimed to comprehensively characterise the HPA response in women to a long and arduous infantry-based basic military training programme in the UK. We hypothesised that compared with the first week of training, ongoing training would be associated with reduced cortisol in the early morning and unchanged or elevated cortisol the preceding evening. Given anticipated effects of sustained psychological stress of adapting to the military environment, intense exercise and restricted sleep, we hypothesised HPA axis responsiveness to ACTH would be reduced, while hair and fasted plasma cortisol would be elevated, and these observations would resolve as training became less arduous.

2. Methods and materials

2.1 Setting

This study is part of the Female Endocrinology in Arduous Training research programme, which comprises studies aiming to characterise female endocrine and metabolic responses to military training. This study took place at the Royal Military Academy, Sandhurst UK, where the British Army trains all Officers during the Commissioning Course. The regular Commissioning Course is an immersive, 44-

week, infantry-based training programme, taking place in mixed sex platoons. It is designed to be physically and mentally arduous, teaching theoretical and practical leadership and the fundamentals of soldiering. The 44-week course is separated into three terms, each 14 weeks long, with 2 weeks of adventurous training.

2.2 Participants, inclusion and exclusion criteria

All women commencing the Commissioning Course over three successive intakes (May 2017, September 2017 and January 2018) were invited to participate at a pre-course briefing held 6 to 20 weeks before the start of training. Immediately before starting the Commissioning Course, cadets underwent a medical examination to confirm fitness, including a detailed medical history, review of medical records and physical examination to exclude among other things medically diagnosed psychological disorders in the past year (including anxiety, depression and eating disorders), treated hormone deficiency (except hypothyroidism, which must have been treated and stable for six months beforehand) and arrhythmia. Exclusion criteria were the use of inhaled, oral or topical steroid preparations in the past three months or during the Commissioning Course. Participation in the study was voluntary, and all women provided informed written consent 24 hours after oral and written briefings. The study was approved by the Ministry of Defence Research Ethics Committee.

2.2 Procedures

The study used a repeated measures design across the three 14-week terms. Study visits took place at the pre-course briefing (visit Pre), beginning and end of term 1 (visits 1 and 2), end of term 2 (visit 3), and end of term 3 (visit 4). Saliva sampling also took place in weeks 5 or 7 of each term **(figure 1).**

2.2.1 Questionnaires

A baseline questionnaire was completed at study visit 1 detailing age, ethnicity, education, and reproductive, medical and surgical history. Five questionnaires were undertaken at the pre-course briefing and the beginning and end of each term: the 10-point Connor Davidson Resilience Scale (CD-

RISC-10) (Connor and Davidson, 2003), patient health-questionnaire 9 (PHQ-9) (Kroenke et al., 2001), psychosocial stress questionnaire of Rosengren et al. (2004), impact of events scale – revised (IES-R) (Weiss, 1997) and the Beck Anxiety Inventory (BAI) (Beck et al., 1988). Questionnaires were completed on smart phones using SmartSurvey (SmartSurvey, Tewkesbury, UK).

The CD-RISC-10 is a measure of the ability to respond to adversity and comprises 10 items, scored from 0 ("not true at all") to 4 ("true nearly all the time"), and is abridged from the 25-point CD-RISC on the basis of a thorough factor analysis (Campbell-Sills and Stein, 2007). The scale has demonstrated strong psychometric properties in young adults (Campbell-Sills et al., 2009) and military populations (Green et al., 2014; Johnson et al., 2011). Permission was granted from the author to use the CD-RISC-10. The PHQ-9 is a measure of low mood, consisting of nine criteria scored from 0 ("not at all") to 3 ("nearly every day"). The PHQ-9 has demonstrated good validity and reliability as a diagnostic and severity measure in military and general populations (Martin et al., 2006; Wells et al., 2013). We analysed scores on a continuous scale of 0 to 27, to detect subtle differences over time, and used the cut-off ≥10 points, which has 88% sensitivity and specificity for moderate depression (Kroenke et al., 2001). The psychosocial stress questionnaire defined stress as feeling irritable, filled with anxiety, or as having sleeping difficulties because of conditions at work or at home, with the following response options: 'never', 'some periods', 'several periods' or 'permanent stress'. In asking about the level of financial stress, three options were given: 'little or none', 'moderate' or 'high or severe', while major life events such as major family conflict, divorce or separation were categorised into 'none' or 'one or more'. Participants were then asked to complete the IES-R with reference to any major life event(s) identified. The BAI assesses how much each of 21 anxiety symptoms has bothered participants in the past month on a 4-point Likert scale from 0 ("not at all") to 3 ("severely – it bothered me a lot"). The BAI has demonstrated high internal consistency in a military population (α coefficient 0.91), adequate test-

retest reliability (r = 0.75) and correlates highly with other measures of anxiety (Beck et al., 1988; Nathan et al., 2012).

2.2.2 Hair sampling

At the pre-course briefing and the end of each term (visits Pre, 2, 3 and 4), a 5 mm diameter section of hair was sampled from the posterior vertex region of the head, as close as possible to the scalp, and stored in aluminium foil at room temperature until transport for analysis by Dresden Lab Service GmbH (Dresden, Germany). Hair samples were divided into 1 cm segments by the laboratory, assuming an average growth rate of 1 cm per month. Up to 7 segments were assayed from visit Pre and four segments from other visits, giving a maximum of 17 1-month hair cortisol concentrations. The number of segments assayed varied according to participant hair length. To account for differing hair lengths, like other studies (e.g. Boesch et al. (2015); McLennan et al. (2016)), we compared average hair cortisol across three-month periods. Participants with hair length ≥ 5 cm and < 5 cm provided five 3-month periods and four 3-month periods, respectively. Subjects using peroxide treatment were excluded from analysis due to its cortisol-lowering effect (Stalder et al., 2017). Due to the negative association of the combined contraceptive pill (CCP) use with hair cortisol (Stalder et al., 2017), CCP users were considered separately from non-users (**see Section 2.4**).

2.2.3 Saliva sampling

Diurnal cortisol slope necessitates morning and evening saliva sampling on the same day, however this was not feasible due to constraints of the training programme. Instead, cortisol was measured from evening and morning saliva samples from saliva samples on consecutive days at the beginning, middle and end of each term. Participants were requested to provide saliva samples using a Sarstedt Salivette ® (Sarstedt, Leicester, UK), by chewing on the synthetic swab for 30 secs, as described elsewhere (Stalder et al., 2016). Saliva samples were collected immediately before bed (before brushing teeth) and immediately after waking the following morning. Sampling instructions were given through live

demonstration, videos and written instructions (on paper and by text message). Participants documented the time of sampling on the tube. Reminders were sent to participants' mobile phones by text message at around 10 pm on the evening of sampling and at around 6 am on the morning of sampling (Cadets normally woke shortly before 6 am).

2.2.4 Basal and dynamic blood sampling

A single sample of blood was collected in EDTA-containing tubes after an overnight fast at visits 1, 3 and 4. Each blood sample was analysed for cortisol binding globulin (CBG), albumin and cortisol. The day after fasted blood sampling on visits 1 and 3, a 1-hour combined dynamic adrenal function test was used to assess adrenal cortex function (Morosini et al., 1989). Due to constraints imposed by training, dynamic testing was completed in the early evening (average time 6.51 pm ± 51 mins, range 5.20 pm to 8.10 pm) and for each participant was completed at the same time on both occasions. Participants relaxed supine on a bed before a 20 G cannula was inserted into an antecubital fossa vein. A sample of blood was taken from the cannula in EDTA-containing tubes. After 10-15 minutes, 1.0 µL of a 1µg/ml solution of adrenocorticotrophic hormone ($ACTH_{1-24}$, tetracosactrin acetate as Synacthen®, Mallinckrodt, Dublin, Ireland), freshly diluted on each occasion as described previously (Gifford et al., 2019), was injected followed by a 10 mL saline flush. Venous blood was sampled from the cannula in EDTA-containing tubes after 20, 30, 40 and 60 min. Basal (afternoon), peak (stimulated), and area under the curve (AUC) dynamic plasma cortisol concentrations were assessed, with AUC calculated using the trapezoidal rule. For plasma cortisol analyses, participants were considered separately if they used a CCP, since synthetic oestrogens would be expected to elevate CBG levels and thus total cortisol. Since sex hormones are expected to alter cortisol responsiveness under psychosocial stress (Stephens et al., 2016) when plasma cortisol was assessed, participants who did not use hormonal contraception were asked the number of days since the first day of their last menstrual period.

2.3 Laboratory methods

Hair cortisol was assayed from 1 cm samples using methods described elsewhere (Iob et al., 2018). Saliva cortisol was assayed using a commercial ELISA kit (Salimetrics ®, State College, PA). Cortisol quantities in the plasma samples were obtained following extraction and LC-MS/MS analysis. Briefly, a calibration curve of cortisol was prepared alongside plasma samples (200 µL) enriched with isotopically labelled cortisol. Samples were extracted using Supported Liquid Extraction SLE400 cartridges (Biotage, UK) by diluting in 0.5M ammonium hydroxide (200 µL), loading, eluting with dichloromethane/isopropanol (0.45 mL x 3), drying under nitrogen and resuspending in 70:30 water/methanol (100 µL described previously (Spaanderman et al., 2018)). Chromatographic separation was achieved following injection (20 µL) using a gradient on a Shimadzu Nexera UPLC system on a Kinetex C18 (150 x 3 mm; 2 µm) column of mobile phases: 0.1 % FA in water, 0.1 % FA in methanol, 0.5 mL/min, 30 °C, followed by MS/MS analysis on a Sciex QTrap 6500+ operated in positive ESI, where Mass Spectrometry settings have been described previously (Stirrat et al., 2018). Least squares regression of the peak area ratio, with 1/x weighting, was used to calculate the amount of steroid in each sample within Analyst MultiQuant software (Sciex, UK). Total CBG was assayed from plasma using ELISA as per Lewis and Elder (2011), and albumin was assayed using commercial kits (Alpha Laboratories, Eastleigh, UK) adapted for use on a Cobas Fara centrifugal analyser (Roche, UK).

2.4 Statistical analyses

Statistical analyses were performed using SPSS 24.0 for Mac (IBM, New York, NY). Data were visually assessed for normality and non-normal data were transformed prior to analysis using parametric tests (CBG and average 3-month hair cortisol concentrations were transformed by natural logarithms). Baseline demographics of participants who withdrew were compared with those who completed the study using independent samples t-tests and χ^2 for continuous and categorical variables, respectively. Four participants were excluded from analyses of hair and plasma cortisol due to commencing or discontinuing a CCP during the study (change in CCP use precluded repeated measures due to the effect of CCP on CBG); a further two were excluded from analyses of hair cortisol due to peroxide

257 treatment. Fourteen participants used a CCP throughout the study. Missing data (saliva cortisol) were
258 imputed using group means for those time points (159 samples, 17%) before analysis of successive
259 morning and evening concentrations.

260 Changes in questionnaire scores and days since last menstrual period were assessed using repeated
261 measures ANOVAs (main effect of time [visit 1 vs visit 2 vs visit 3 vs visit 4]), with *post-hoc* uncorrected
262 paired samples t-tests used to assess differences between time-points in the event of a significant main
263 effect. Where statistically significant changes in questionnaire score were identified, scores for
264 individual questions within those questionnaires were compared over time using RM ANOVA with
265 Bonferroni adjustment. Changes in hair and saliva cortisol concentration were assessed using a two-
266 way mixed-design ANOVA (group [CCP user vs non-CCP user] × time). Changes in dynamic cortisol
267 concentration from visit 1 to visit 3 were assessed using paired samples t-tests; comparisons between
268 CCP users and non-CCP users were made using independent samples t-tests. A p-value <0.05 was
269 deemed significant.
270

271 ## 3. Results

272 ### 3.1 Participant characteristics

273 Of 77 women who attended the study briefing, 68 (88%) volunteered to participate **(figure 2)**. Five
274 participants (8%) completed the baseline visit (visit Pre) but did not commence the Commissioning
275 Course. Ten (15%) withdrew during the Commissioning Course: six during term 1 (two medically
276 discharged on arrival, three due to training-related injury, one chose to withdraw from the study), two
277 during term 2 (training-related injury), and two during term 3 (training-related injury). A total of 53
278 women completed the study; their baseline characteristics compared with participants who withdrew
279 are presented in table 1. The age, rate of stressful events and anxiety scores did not differ between
280 those who withdrew and those who completed the study. There were no correlations between cortisol
281 indices with age, ethnicity or educational qualification.

282

283 ## 3.2 Procedures

284 ### 3.2.1 Questionnaires

285 Questionnaires scores and statistical significance indicators are presented in table 2. CD-RISC-10 and
286 PHQ9 scores decreased and increased, respectively, across the Commissioning Course with modest to
287 large effect sizes. Post-hoc tests showed significant decreases from visit Pre at all visits (1 to 4) for CD-
288 RISC-10, and increases from visit Pre at visits 2 to 4 for PHQ-9. Question-by-question analysis of CD-
289 RISC-10 (**supplementary material A**) showed modest decreases in measures of traits labelled
290 'hardiness', specifically the ability to cope with change and illness, injury and hardship, and
291 'persistence', specifically not giving up and working to attain goals despite roadblocks (Campbell-Sills
292 and Stein, 2007). For the PHQ-9, subsequent analysis showed a significant increase in all domains
293 except concentration and psychomotor function, which were elevated throughout the study
294 (**supplementary material B**) (Kroenke et al., 2001). Forty participants (74%) reported 'feeling tired or
295 having little energy' for 'several days or more' throughout the study, while the number reporting 'feeling
296 tired or having little energy' increased significantly from visit 1 to visits 2 to 4, being highest at visit 2 (49
297 (92%) reported this "several days" or more). Twelve participants (18%) reached the PHQ-9 cut-off (≥10
298 points) on one occasion and 4 (6%) on two occasions. Of these participants, ten (83%) also described
299 a stressful life event not related to the training (e.g. death of a loved one or divorce), which may
300 account for higher scores suggesting low mood. More participants described feeling work-related stress
301 (feeling irritable, filled with anxiety, or as having sleeping difficulties) over several periods or
302 permanently during the Commissioning Course compared with before training, and this finding was
303 most marked at visits 2 and 3. Anxiety scores did not change during the study, although the number of
304 participants reporting financial stress and stress due to work increased from visits 1 to 3, and 3 to 4
305 (table 2).

306 ### 3.2.2 Hair cortisol concentration

Monthly hair cortisol concentrations are shown in figure 3A and comparisons of 3-month average hair cortisol concentrations between CCP and non-CCP users are displayed in table 3. There was no CCP use × time interaction for hair cortisol, but the effect of time was significant (p=0.003, table 3) demonstrating that hair cortisol increased in both non-CCP users and CCP users. Post-hoc t-tests demonstrated hair cortisol was higher at months pre-3 to pre-1 and months 1 to 4 and 9 to 12 of training than months pre-6 to pre-4 (table 3).

3.2.3 Saliva cortisol concentration

Evening and morning saliva sampling recording times were 11.12 pm ±35 min and 6.07 am ±28 min, respectively. Evening saliva concentrations did not change during the Commissioning Course (figure 3B). There was a main effect of time for morning cortisol (p<0.001), with post-hoc t-tests demonstrating that morning cortisol increased from week 1 to week 7 of term 1 (0.44 ±0.23 versus 0.59 ±0.24 µg/dl, p<0.001, figure 3B), with no significant differences between any other time-points. Morning salivary cortisol in term 1 week 1 was not different to term 3 week 13 (0.44 ±0.23 versus 0.38 ±0.21 µg/dl, p=0.2). The response of CCP users was not different to non-CCP users (group × time interaction, p=0.4).

3.2.4 Basal and dynamic blood tests

Cortisol binding globulin was higher among CCP users than non CCP users (median (interquartile range) at visit 1: 379 (165, 444) ng/ml versus 95 (63, 220) ng/ml, respectively at visit 1, p<0.001) but did not change in either group during the Commissioning Course (p=0.6, Supplementary material C). Albumin did not differ between CCP users and non-users (34.3 ±2.3 versus 35.0 ±2.6 g/l, respectively at visit 1, p=0.6) and did not change during the Commissioning Course (p=0.7, supplementary material C). In non-CCP users, fasting plasma cortisol decreased progressively from visits 1 to visits 3 and 4 (701 ±134, 671 ±158 and 561 ±177 ng/ml, respectively, p<0.001, figure 3C), with significant post-hoc differences in non-CCP users between visit 1 and visits 3 and 4 (p=0.009 and p<0.001, respectively,

figure 3C and supplementary material C). By contrast, in non-CCP users, dynamic function testing demonstrated an increase in afternoon basal cortisol from visits 1 to 3 (177 ±92 and 259 ±103 nmol/l, respectively, p=0.003; figure 3D and supplementary material C) and suggested an increase in peak stimulated cortisol (589 ±164 and 656 ±135 nmol/l, p=0.058, figure 3D and supplementary material C). Fasting plasma cortisol decreased in CCP users from visit 1 to visit 4 (1065 ±193 nmol/l versus 859 ±186* nmol/l, p=0.013, figure 3C and supplementary material C). There was no effect of CCP use for fasting cortisol, (CCP use × time interaction, p=0.9, figure 3C and supplementary material C) and in CCP users afternoon cortisol, peak cortisol response to ACTH and cortisol AUC did not change from visit 1 to visit 3 (figure 3D and supplementary material C). In participants not using hormonal contraceptives, duration of days since last menstrual cycle did not differ between visits 1, 4 and 6 (19 ±19 days,16 ±12 days and 15 ±10 days, respectively, p=0.5).

4. Discussion

This study comprehensively characterised the HPA axis response to prolonged arduous infantry-based military training in women. We demonstrated a significant rise in morning salivary cortisol concentrations from week 1 to 7 of training, tending to suggest a normal stress response, which is in contrast to the relative decrease in morning cortisol, which we had hypothesised; evening saliva cortisol did not change. Thereafter, saliva cortisol concentrations appeared to demonstrate habituation, returning to baseline levels by the end of training, corroborated by a decrease in morning fasting plasma cortisol. Peak stimulated cortisol rose modestly (in non-CCP users), suggesting the training was associated with a slight increase in HPA axis responsiveness. Average cortisol concentration in hair demonstrated a modest rise during training.

In our study design we were unable to obtain a true baseline saliva cortisol; participants were already 3 days into training when testing started, so the first sample may have reflected some of the ‘shock of

355 capture'. However, our findings of habituation through training are perhaps consistent with those of
356 Clow et al. (2006), who found a latent decrease in cortisol awakening response during 11-week basic
357 military training in male and female British Army recruits. The increase in hair cortisol measured before
358 the course lies within known rates of cortisol washout (29% loss from the most proximal 3 cm to the
359 next most proximal 3 cm segment, from the meta-analysis by Stalder et al. (2017)), so we are unable to
360 determine if there was a true anticipatory rise prior to training. The rise in hair cortisol observed during
361 the Commissioning Course is contrary to Boesch et al. (2015), who found no change in male hair
362 cortisol during training in a single intake of Swiss military cadets. However, Boesch et al. (2015)
363 highlighted shortcomings of their study including the inability to obtain long enough hair samples, which
364 resulted in relatively short hair cortisol exposures, which were interrupted by haircuts, pretraining hair
365 cortisol concentration and affected by seasonal variation. The use of women in our study helped
366 overcome this, while our recruitment over three courses meant pre- and within-training hair cortisol
367 concentrations represented continuous exposures of 27 months. While average hair cortisol
368 concentration did not exhibit the same HPA habituation seen in the morning saliva cortisol (a stress
369 response), the increase throughout training may be explained by regular physical exercise during
370 training (Gerber et al., 2012); chronic stress but not self-report measures of percieved stress could be
371 expected to elicit increased hair cortisol (Stalder et al., 2017). In a study of six women undertaking an
372 extremely arduous transantarctic ski expedition, hair cortisol was markedly elevated throughout the
373 expedition (Gifford et al., 2019), which accords with other studies of athletes (reviewed in Gerber et al.
374 (2012), but psychological stress scores were reduced and resilience scores were unchanged. While in
375 the current study, resilience and mood decreased while hair cortisol increased, a recent meta-analysis
376 found no association between various scales of low mood and hair cortisol ($r=-0.059$, $p=0.078$) (Stalder
377 et al., 2017). We conclude the rise in hair cortisol was more likely a reflection of physical activity or
378 energy deficit, than low mood or psychological stress.

Contrary to our hypothesis, we demonstrated a concurrent increase in the plasma cortisol response to ACTH with decreased early morning plasma cortisol. In a similar dynamic function test, veterans with traumatic military experiences demonstrated increased responsiveness to ACTH compared with controls, which was unrelated to anxiety disorders (Golier et al., 2014). Pre-stimulation morning cortisol levels are often elevated while stimulated cortisol may be suppressed in overtraining syndromes (Cadegiani and Kater, 2017). In our previous study of women undertaking an arduous ski expedition, responsiveness to a similar 1μg ACTH test was suppressed, with marked sensitivity to central negative feedback, but did not change immediately following or two weeks after a 2 month exercise exposure, compared with 1 month beforehand (Gifford et al., 2019). In the present study, the increase in cortisol responsiveness was not accounted for by changes in CBG. We postulate our findings represent an increased HPA axis responsiveness during training which could be interpreted as 'healthy'.

Resilience scores were consistent with the upper end of the range reported previously for similar populations throughout the study (Davidson, 2018), despite demonstrating a modest but steady decrease during training. The slight decrease in resilience constituted reduced hardiness and persistence ratings, which could be related to fatigue. Certainly, the PHQ-9 scores may have been distorted by a lack of sleep. For example, the question 'do you have trouble falling or staying asleep, or sleeping too much' was perhaps confounding, since it was more likely to reflect a tiring training programme than low mood. Where the clinical cut-off of the PHQ-9 was reached (≥10 points), this was generally attributable to a non-course related adverse event, which likely explained the overall increase in PHQ-9 (although the number reporting work-related stress increased from 7 in term 1 to 10 in term 3). Alternatively, it is possible that the changes observed in CD-RISC-10 and PHQ-9 related to the increased ratings of stress from work.

Strengths of our study include the multimodal approach to HPA axis assessment, alongside repeated measures of mood and resilience and the large sample of female military cadets studied during

arduous training over a long duration. Participants were well-matched and were undertaking an identical arduous training programme, which will be relevant to women in physically demanding occupations.

Unfortunately, we were limited to diurnal cortisol measurement and were unable to examine cortisol awakening response due to restraints on the participants' time (they were often undertaking programmed activities within 1 hour of waking) and our saliva cortisol findings are, therefore, preliminary. We were also unable to perform dynamic HPA axis testing in the morning, so could not assess central axis sensitivity to dexamethasone to determine whether there were any changes in central negative feedback sensitivity (Reynolds et al., 2001a). The Course was a relatively long military training programme; shorter duration training, which is more common, might provoke pathological activity of the HPA in female military cadets. Therefore, the findings of the present study need to be replicated by further studies providing a different training content to enhance the generalisability of the results.

Our hypothesis that military women would demonstrate maladaptive cortisol responses to basic military training was rejected. Through a comprehensive assessment, the initial rise in morning cortisol and fasting plasma cortisol, appeared to be followed by habituation, and increased HPA axis responsiveness. These responses were observed despite modest reductions in mood and resilience and increased perceived stress during training. The observed increase in hair cortisol during training was possibly related to physical exercise. We interpret these findings as being consistent with a healthy adaptation of the HPA axis during basic military training among women, despite evidence of ongoing perceived stress.

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433

Table titles, descriptions, footnotes

Table 1.

Title: Demographics

Footnote: Data are mean ± Standard deviation, ns: p>0.10, IES-R: impact of events scale – revised, CD-RISC-10: Connor Davidson Resilience Scale-10, PHQ-9: patient health-questionnaire, BAI: Beck Anxiety Inventory.

Table 2.

Title: Psychological health questionnaires

Footnote: Data are mean ± Standard deviation, ns: p>0.10. IES-R: impact of events scale – revised, CD-RISC-10: Connor Davidson Resilience Scale-10, PHQ-9: patient health-questionnaire, BAI: Beck Anxiety Inventory. RM ANOVA Repeated measures analysis of variance. At visits pre- and visit 1 this question was 'how often have you experienced stress due to school, university or work?' * post hoc p<0.05 for paired t-test versus visit Pre.

Table 3.

Title: Average hair cortisol concentrations in 3-month periods

Footnotes: Combined contraceptive pill (CCP, n=13) users and non-CCP users (n=33) considered separately due to known association of CCP use with hair cortisol. Data are median (interquartile range). p value for two-way repeated measures ANOVA (main effect of time or group × time). * post hoc p<0.05 for paired t-test versus months pre-6 to pre-4.

Figure captions.

Figure 1. Schematic of study visits. Study visits (numbered Pre, 1, 2, 3 and 4) and saliva sampling (indicated by *) are indicated below the weeks in which they took place. PCCBC, pre-Commissioning Course Briefing Course

Figure 2. Recruitment and follow-up.

Figure 3. A: One-month average hair cortisol concentrations prior to and during the Commissioning Course (all participants); month 'pre' was prior to the Course starting. Hair was sampled at study visits 'Pre' (either month Pre 1 or Pre 2), 2 (month 4), 4 (month 8) and 6 (month 12). **B: Evening and morning saliva cortisol concentrations;** top panel: sampled in the evening, bottom panel: sampled the following morning. **C: Fasting plasma cortisol concentration;** non-CCP users (black column) and CCP users (grey column)**. D: Mean ± SD total cortisol concentrations during dynamic 1-25 ACTH testing;** non-CCP users (left, n=39) and CCP users (right, n=13) at visit 1 (filled square) and visit 3 (unfilled square). **Legend.** Data are mean ± SD. Solid bracket: mixed two-way ANOVA, Dotted line: significant post-hoc comparisons. ******* $p<0.001$, ***** $p<0.05$, ns $p>0.10$. **** (1)* $p<0.001$ for effect of time; no interaction of group [CCP users vs non-CCP users] × time.

No requirement for colour figures

Supplementary Material:

Supplementary Material A

Supplementary Material B

Supplementary Material C

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627

Fig 1

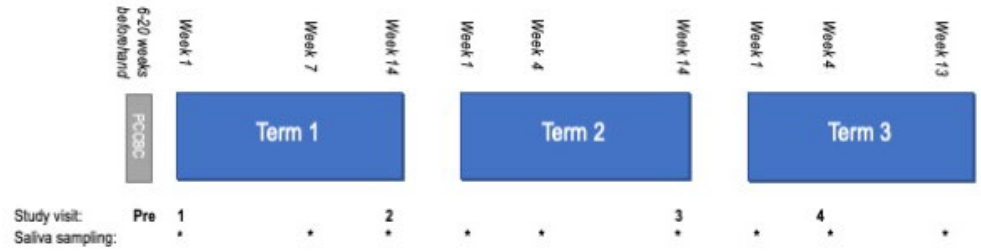

Fig 2

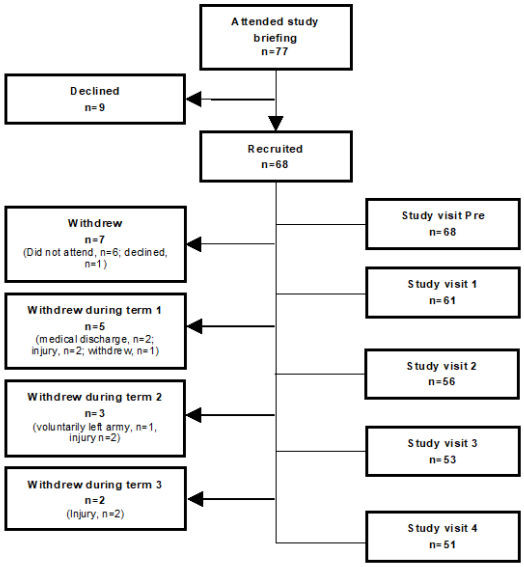

Fig 3

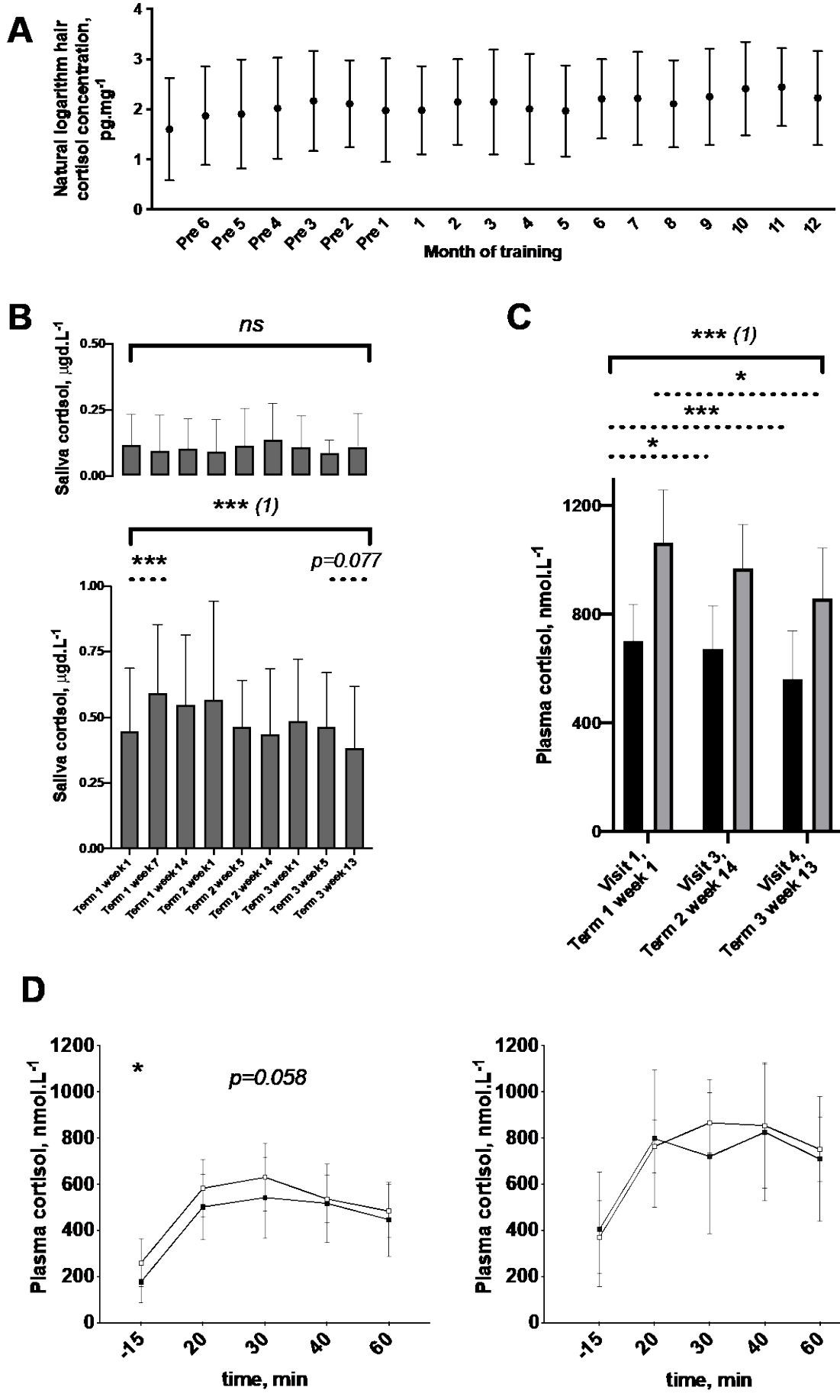
A
Natural logarithm hair cortisol concentration, pg.mg-1
Month of training
Pre 6
Pre 5
Pre 4
Pre 3
Pre 2
Pre 1
B
ns
Sallva cortisol, µgd.L-1
*** (1)
***
p=0.077
Term 1 week 1
Term 1 week 7
Term 1 week 14
Term 2 week 1
Term 2 week 5
Term 2 week 14
Term 3 week 1
Term 3 week 5
Term 3 week 13
C
*** (1)
Plasma cortisol, nmol.L-1
Visit 1, Term 1 week 1
Visit 3, Term 2 week 14
Visit 4, Term 3 week 13
D
p=0.058
time, min

Table 1. Demographics

	Completed the study (n=52)	Withdrew (n=18)	p
Age, years	24.0 ±2.5	23.9 ±2.8	ns
Ethnicity, n (%)			
White Scottish. English/ Welsh/ Northern Irish/ British	50 (96)	18 (100)	ns
White Irish	1 (2)	0 (0)	
Other white background	1 (2)	0 (0)	
Highest educational qualification, n (%)			
Master's degree	10 (21)	3 (17)	
Bachelor's degree	36 (70)	9 (50)	
Secondary school	6 (11)	6 (33)	ns
Smoker, n (%)	3 (6)	2 (12)	ns
Drink alcohol (yes) n (%)	48 (91)	12 (80)	ns
Age at menarche, years; median (range)	13 (11-16)	13 (11-16)	ns
Contraception			
Combined contraceptive pill	13 (25x)	5 (29)	
Progestogen-only	16 (32)	4 (24)	
None or intrauterine device	18 (34)	8 (47)	
Discontinued combined contraceptive pill during study	3 (5)		
Commenced combined contraceptive pill during study	1 (4)		ns
Several periods of psychological stress, n (%)	14 (26)	1 (9)	ns
Permanent, psychosocial stress, n (%)	0 (0)	0 (0)	
Some periods of psychological stress, n (%)	35 (66)	13 (87)	
Never experienced psychological stress, n (%)	4 (8)	1 (9)	
High or severe financial stress, n (%)	1 (2)	0 (0)	-
One or more adverse events, n (%)	17 (32)	3 (27)	ns
IES-R with respect to adverse event	12 ±11	6 ±1	ns
CDRISC 10	30 ±5	30 ±3	ns
PHQ-9	4 ±3	3 ±4	ns
BAI	8 ±7	6 ±3	ns
Peroxide hair treatment	2 (4%)	0 (0)	-
Hair cortisol concentration, 4 to 6 months before Course, median (interquartile range), pg/mg	5.95 (3.53, 13.9)	6.77 (1.91, 15.5)	ns
Hair cortisol concentration, 1 to 3 months before Course, median (interquartile range), pg/mg	8.65 (5.22, 15.4)	5.50 (2.98, 11.9)	ns

Table 2. Psychological health questionnaires

	Visit Pre	Visit 1	Visit 2	Visit 3	Visit 4	RM ANOVA F	p
CD RISC 10							
	32.6 ±4.1	30.2 ±4.9*	29.3 ±5.5*	30.4 ±5.6*	29.2 ±5.5*	6.93	<0.001
PHQ-9							
	3.1 ±3.0	3.4 ±2.6	5.5 ±3.5*	4.6 ±3.9*	4.6 ±4.4*	7.24	<0.001
Number reaching cut-off score of 10, n (%) 1 (2)		2 (4)	5 (9)	5 (9)	7 (13)		
Adverse events and IES-R							
	6 events in 6 participants 24.8 ±15.4	8 events in 8 participants 13 ±10.49	10 events in 10 participants 25.7 ±15.2	11 events in 9 participants 24.0 ±16.1	9 events in 9 participants 25.5 ±15.7		
BAI							
	7.7 ±6.7	6.9 ±5.2	6.3 ±5.8	6.1 ±6.3	5.2 ±6.2	1.10	ns
High or severe financial stress, n (%)							
	1 (1)	2 (3)	2 (3)	5 (9)	4 (8)		
How often have you experience stress due to work?* , n (%)							
Never Some periods Several periods Permanently Not working or at school or university	3 (6) 35 (66) 15 (28) 0 (0) 5 (9)	6 (11) 22 (41) 17 (32) 1 (2) 7 (13)	0 (0) 17 (32) 33 (62) 3 (6) 0 (0)	0 (0) 23 (43) 25 (47) 5 (9) 0 (0)	0 (0) 25 (47) 23 (43) 5 (9) 0 (0)		

Table 3. Average hair cortisol concentrations

	Months pre-6 to pre-4, pg/mg	Months pre-3 to pre-1, pg/mg	Months 1 to 4, pg/mg	Months 5 to 8, pg/mg	Months 9 to 12, pg/mg	
Non CCP users	7.12 (3.88, 13.90)	9.63 (5.38 ,16.26)*	9.60 (6.80, 15.3)*	10.39 (5.56, 17.28)	11.56 (6.20, 22.45)*	F=4.247
CCP users	4.84 (3.52 ,12.99)	6.23 (4.64 ,12.23)*	7.76 (4.08, 11.26)	10.08 (7.27, 13.36)	13.7 (6.1, 18.63)*	F = 3.236
Effect of CCP use × time						ns
Effect of time						p=0.003