Sex-related Hypothalamic-Pituitary-Gonadal and Hypothalamic-Pituitary-Adrenal Axis adaptation during Military Training

Running title: Sex Differences in Endocrine Adaptation to Military Training

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Abstract

Reproductive endocrine function adapts to psychological, environmental, and energy-associated stressors. Multi-stressor environments upregulate hypothalamic-pituitary-adrenal (HPA) axis, causing suppression of the hypothalamic-pituitary-gonadal (HPG) axis, but it is not known if this pattern or its magnitude is sex-biased. We compared HPG and HPA axis activity in 9 men and 34 women undergoing Army training. 1-hour low-dose Gonadorelin and SynACTHen tests were conducted at 1 and 29 weeks, measuring gonadotrophins and cortisol. Cortisol was measured from hair every three months. Morning and evening salivary cortisol and psychometric questionnaires were measured at six timepoints. Sexes were compared over time by two-way ANOVA. Gonadotrophin responses were significantly higher in women than men in week 1, but no sex difference was seen at week 29 (no significant sex × time interaction). Week 1 cortisol response was higher among men, but week 29 cortisol response was higher among women (sex × time $F_{(1,44)}=18.0$, $p<0.001$). Hair cortisol was higher among women than men beforehand, not different between sexes during the first three months, and significantly higher among women during training months 5-11 ($F_{(3,15)}=3.25$, $p=0.024$). Morning salivary cortisol was higher among women in week 8 and week 14, but higher among men in week 29 ($F_{(4,76)}=4.0$, $p=0.005$). No differences were seen in evening salivary cortisol. Psychometrics did not change or differ between sexes. HPA axis responses to military training were greater among women than men. HPG axis responses suggest greater downregulation among women. These findings will enable equitable and individualised management of people undergoing periods of intensive physical stress.

New and noteworthy: We conducted a comprehensive comparison of adrenal and reproductive function in men and women undergoing 11-month military training. We found progressively elevated cortisol levels and dynamic cortisol response to stress among women, but not men, and suppression of reproductive function among women. The physiological impact of stressful military training was greater among women than men; this could not be explained by energy balance, and sex-specific effects of sleep, socio-ethnographic or other stressors may be responsible.

1 Introduction

Regulation of reproductive function is dynamic and adaptive. External factors such as psychological stress, environmental challenge, and availability of energy can be considered 'stressors,' which activate the hypothalamic-pituitary-adrenal (HPA) axis, increasing production of the primary human glucocorticoid, cortisol. Activation of the HPA axis suppresses the hypothalamic-pituitary-gonadal (HPG) axis (1-4).

The HPA axis demonstrates sex-biased activity. Gonadal steroids are known to modulate HPA axis reactivity; circulating oestrogens enhance the glucocorticoid response to stress, increasing adrenal sensitivity to adrenocorticotrophin (ACTH) (5, 6), while androgens have the opposite effect, attenuating the HPA axis response to a stressor (7) by dampening adrenal responsiveness (8). Adolescent girls demonstrate greater sensitivity to ACTH-(1-24) than boys (9). Sexual dimorphism in stress processing has also been observed in the absence of gonadal steroids. Following pharmacologically induced HPG axis suppression, enhanced ACTH and cortisol responses to corticotrophin-releasing hormone (CRH) and exercise were observed among men compared with women (10). However, the sex difference in adrenal responsivity was attenuated, suggesting neural regulation of the HPA axis and central feedback mechanisms may be sexually dimorphic (10), perhaps reflecting differing energetic requirements (11). HPA axis function is also moderated by cortisol binding globulin (CBG) (12), the production of which is governed by oestrogens, and arginine vasopressin (AVP), both sexually dimorphic in their abundance.

Clinical studies seeking to understand the effect of exercise and its associated stressors on reproductive function demonstrate that insufficient caloric intake relative to exercise energy expenditure is a primary stressor which can lead to suppression of the HPG axis, manifesting clinically as hypothalamic amenorrhoea (13). While clinical manifestations of HPG axis suppression are likely to be more apparent in women than men, a recent review suggests men may be less susceptible to these effects (13).

We have previously reported that women undertaking arduous military training demonstrated anovulation and significant suppression of gonadotroph function (14) and activation of the HPA axis (15). Men undertaking the same training demonstrated significantly greater energy deficits than women, due to higher energy expenditure

(16). We aimed to compare dynamic HPG and HPA axis activity between men and women from these studies. We hypothesised that exposure to military training would produce a sex-biased HPG and HPA axis change, with enhanced responsiveness and basal levels of cortisol, with attendant suppression of HPG axis function, among women compared with men.

2 Methods

2.1 Participants

Participants were training to enter the British Army as Officers at the Royal Military Academy, Sandhurst, UK. This course provides comprehensive physical, mental, and environmental challenges over three, fourteen-week terms separated by two or three weeks of leave, as described previously (14, 17). Officer Cadets undergo competitive selection for roles within the army during week 20. Training is undertaken in mixed-sex groups; the proportion of female Officer Cadets is typically around 8-12%.

The Female Endocrinology in Arduous Training cohort study examined endocrine, metabolic, and bone health in female Officer Cadets over three successive Commissioning Course intakes. Findings from female participants demonstrated high levels of physical activity,(17) activation of the HPA axis(15) and suppression of the HPG axis with maladaptive metabolic changes(14) over 44 weeks of training, with temporary uncoupling of bone turnover.(18) A cohort of men was studied during one intake. We have previously demonstrated more negative energy balance among these men than female contemporaries.(16) Here we present a sex comparison of HPA and HPG axis changes. Inclusion criteria were: medically fit to commence the course and aged 18-30 years. Participant health status was confirmed by entry medical examination prior to enrolment, including history, examination and ECG, completed according to entry requirements for UK Defence (19). Exclusion criteria were pregnancy, known history of adrenal, gonadal or GnRH insufficiency, pituitary disease, thyroid disease in the past year, diabetes, hyperparathyroidism, osteopoenia, oral, inhaled or topical glucocorticoid use or ongoing musculoskeletal injury. Due to known effects of synthetic oestrogens on CBG, total cortisol, hair cortisol, LH and FSH, combined oral contraceptive pill (COCP) users were excluded from this analysis (9, 14, 20). Participants who withdrew from or did not commence training (n=10 and 7, respectively) or from the study measures (0 participants)

were excluded from the analysis. All participants provided informed consent. Ethical approval was obtained from the Ministry of Defence Research Ethics Committee.

Experimental design

Participant body mass and height were measured and a questionnaire was completed which ascertained age, contraceptive use, the occurrence of any stressful life events in the past month, levels of financial and work stress, Impact of Events Scale – Revised (IES-R)(21) with relation to any stressful events, Patient Health Questionnaire 9 (PHQ-9) (22), Beck Anxiety Inventory (BAI) (23), and Connor Davidson Resilience Scale 10 (CDRISC 10) (24). In weeks 1, 14, 29 and 44 of training, PHQ-9, BAI, CDRISC 10, significant personal stressors and IES-R, and levels of work and financial stress were recorded.

A simultaneous, low-dose gonadotrophin releasing hormone (GnRH) and ACTH test was used to detect differences in cortisol, LH and FSH responsiveness (25) at week 1 and 28 of training. Due to constraints imposed by the training schedule, dynamic testing was completed in the late afternoon, and testing was not synchronised to menstrual cycle phase. Participants were allowed to relax before a 20G 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, 10µg gonadorelin hydrochloride (Intrapharm, Maidenhead, UK) followed by 1.0µg ACTH-(1-24) (Synacthen®, Mallinckrodt, Dublin, Ireland) was injected followed by a 10mL saline flush. Sampling was repeated after 20, 30, 40, and 60 minutes. Venous blood was also sampled after a 10 hour fast in weeks 1, 14, and 29. Blood was centrifuged and stored at –80℃ until analysis. Saliva was sampled using a synthetic swab (Salivette®, Sarstedt, Leicester, UK), during weeks 1, 8, 14, 16, 20, and 29, before bed in the evening followed by first thing the following morning. Saliva was sampled before brushing teeth and participants were given verbal, written, and video instructions on the technique. Saliva was stored at 5℃ for up to 3 days prior to being stored at –80℃ until analysis. A 5mm diameter section of hair was sampled from the scalp at the posterior vertex region at the start of the study, and at weeks 14 and 29. Hair was stored in aluminium foil at room temperature until analysis.

2.3 Laboratory methods

LH and FSH were assayed by Abbott Architect ® (Abbot, Longford, Ireland). Plasma cortisol was extracted by supported liquid extraction using the Biotage Extrahera robot (Biotage AB, Stockholm, Sweden) and measured

using tandem liquid chromatography mass spectrometry (LCMS/MS) as described previously (15). Total CBG was assayed from plasma using immunoassay according to the method of Lewis and Elder (26). Cortisol was assayed from saliva using a commercial immunoassay according to manufacturer's instructions (Salimetrics®, State College, PA). Hair cortisol was assayed in 1 cm segments, assuming an average growth rate of 1 cm per month (27), by Dresden Lab Service GmbH (Dresden, Germany) using LCMS/MS as described previously (28), providing average 1-month cortisol exposure. Coefficient variations were <4% for Architect assays and <10% for immunoassays.

2.4 Statistical analysis

Data were assessed for normality using the Shapiro Wilk test. Normally distributed data are presented as mean ±SD and non-normal data as median (IQR). Peak fold-wise increases in LH and FSH from prior to GnRH administration, and 1-hour fold-wise increase area under the curve (AUC, calculated by the trapezoidal rule) were calculated. Data from female progesterone-only contraceptive users and non-contraceptive users were pooled for analyses, since no differences were seen between these groups in foldwise LH or FSH responses (Table 3). Missing data were excluded (97 saliva samples (18.8%) due to insufficient volume for analysis). No values were reported below the level of quantification and no data were imputed. The peak and 1-hour AUC of cortisol response to ACTH-(1-24) administration were calculated. Hair cortisol concentrations were analysed using individual mean concentrations over four consecutive 3–4-month periods, to account for differing hair length (4 months pre, and three subsequent 3–4-month periods of training). Peak and AUC fold-wise LH and FSH, peak and AUC plasma cortisol, hair cortisol, PHQ9 and BAI data were transformed by base *e* (Ln).

Male and female physical and psychological characteristics at baseline were compared using independent samples t-tests. Sex differences in CDRISC-10, IES-R, and Ln-transformed PHQ9 and BAI were assessed over time using mixed, repeated measures ANOVA. Ln-transformed fold-wise peak and AUC of LH and FSH response to GnRH, peak and AUC of plasma cortisol response to ACTH-(1-24), CBG and fasted plasma cortisol were compared across groups from week 1 to week 29 by two-way ANOVA (sex × time). Post-hoc comparisons compared sexes at each timepoint (independent samples t-test). Male and female Ln-transformed average hair

cortisol and morning and evening salivary cortisol concentrations were compared over time by mixed repeated measures ANOVA.

Statistical analyses were conducted in SPSS for mac, version 29.0 (IBM, New York, USA). Significance was set at $p<0.05$.

3 Results

A total of 78 Officer Cadets volunteered for the study (68 female, 10 male), of whom 61 completed the study (7 did not commence training (all female), 10 withdrew from training (9 female, 1 male)) and 18 were excluded from this analysis due to COCP use. A breakdown of contraceptive use among included participants is shown in Table 1. A complete dataset is presented for 43 participants (34 female and 9 male).

3.1 Physical and psychological characteristics

Physical and psychological characteristics are shown in Table 1. Men were significantly taller and heavier than women, resilience levels were robust, and scores of anxiety and depression were low, with no differences between sexes. Women had experienced numerically more adverse events in the month prior to training than men. Levels of financial and work-related stress were similar between sexes. During training, scores of depression (PHQ-9) increased from week 1 to weeks 14 and 29, and anxiety (BAI) increased marginally at week 14, while resilience (CDRISC-19) did not significantly change. No sex by time interactions were seen (Table 2). The number of adverse events increased from week 1 to 29 in women but not in men; the stressor impact score (IES-R) associated with these events increased from week 1 to week 29 but did not differ between men and women. Work-related and financial stresses was similar between the sexes.

3.2 LH and FSH

The median (IQR) duration from the first day of the last menstrual period to the GnRH test was 16 (7, 20) and 16 (8, 20.5) days, for the week 1 and week 29 tests respectively ($p = 0.78$). Gonadotroph responses to GnRH are shown in Figure 1. While visual trends suggest a greater decrease in female responses after 29 weeks than male, no statistically significant sex by time interaction was seen for FSH (AUC $F_{(1, 60)} = 0.6$, $p = 0.40$; Peak: $F_{(1, 60)} = 0.81$, $p = 0.30$) or LH (AUC $F_{(1, 60)} = 1.0$, $p = 0.30$; Peak $F_{(1, 60)} = 2.0$, $p = 0.15$). There was also no overall change over

time in FSH response (AUC $F_{(1, 60)}$ = 3.27, p = 0.07; Peak: $F_{(1, 60)}$ = 1.65, p = 0.20) although peak LH response decreased (AUC: $F_{(1, 60)}$ 3.52, p = 0.06; Peak: $F_{(1, 60)}$ = 0.8, p = 0.03). In week 1 the response to GnRH was higher among women than men, both FSH (AUC t = −2.92, p = 0.01; Peak: t = −3.04, p = .002) and LH (AUC t = −2.48, p = 0.009; Peak t = −2.6, p = 0.007) but there was no significant difference in week 29 (AUC FSH: t = −1.68, p = 0.06; Peak FSH: t = −1.52, p = 0.07; AUC LH: t = −0.67, p = 0.20; Peak LH: t = −0.64, p = 0.30). A subgroup analysis also showed no differences in FSH or LH responses between progesterone-containing contraceptive users and non-users (Table 3).

3.3 Plasma cortisol exposure in male and female cohorts during training.

Cortisol responses to ACTH are shown in Figure 2. At week 1, cortisol response was greater in men than women, but was higher at week 29 among women than men (sex × time interaction; peak: $F_{(1, 44)}$ = 17.8, p <0.001; AUC: $F_{(1, 44)}$ = 18.0, p <0.001). CBG did not differ significantly between women and men at week 1 (686 ±123 nmol/L versus 753 ± 173 nmol/L, p = 0.40) or week 29 (594 ± 311 nmol/L versus 385 ± 172 nmol/L, p = 0.06); there was no interaction of sex × time ($F_{(1,42)}$ = 1.15, p = 0.30). Total fasted plasma cortisol did not differ between women and men at week 1 or week 29 (686 ± 123 nmol/L versus 753 ± 173 nmol/L p = 0.20, and 662 ±164 nmol/L versus 670 ± 99 mmol/L, respectively, p = 0.90) with no interaction of sex × time ($F_{(1, 44)}$ = 0.24, p = 0.60).

Hair cortisol concentration varied according to sex (sex × time $F_{(3, 15)}$ = 3.25, p = 0.024) being higher among women in the months prior to training, with no significant sex difference seen during the first three months, and higher concentrations among women during months 5-7 and 9-11 of training (Figure 3A). Incomplete salivary cortisol samples were obtained from male participants in week 14 so this timepoint was excluded from data analysis. Morning salivary cortisol varied during training according to sex (sex × time $F_{(4, 76)}$ = 4.00, p = 0.005) being higher among women in week 8, but higher among men in week 20 (a week when significant psychological pressure was induced), with no differences at other times (Figure 3B). No differences were seen in evening salivary cortisol during training or by sex (sex × time $F_{(4, 80)}$ = 1.1, p = 0.30; Figure 3C).

4 Discussion

To the best of our knowledge this is the first study to compare dynamic endocrine function in men and women during arduous military training. Whilst both men and women experienced elevated cortisol levels during training, there was a notable sex biased impact in the magnitude and pattern of HPA axis activation. The initial response of cortisol to ACTH-(1–24) was greater among men, commensurate with expected sex differences in stress responses (29), but after 29 weeks of physical challenge, the response among women was enhanced while the response in men was decreased. Average hair cortisol concentrations increased in women throughout the study and were significantly higher than men before the study and during the latter 4 to 11 months of training. Hair cortisol concentrations are expected to be higher among men in comparison with women on average (30). Morning salivary free cortisol was higher among women during the first 14 weeks of training suggesting greater anticipatory stress (31). However, contrary to our hypothesis, the female gonadotroph response was not significantly suppressed compared with men after 29 weeks (assessed as the interaction between sexes over time), although post-hoc tests demonstrated a reduction in gonadotroph response over time in women, but not men.

Arduous military training imposes significant mental and physiological stress on individuals, driving an adaptive HPA axis response to mobilise energy (15, 32). In studies of military training, HPA axis upregulation and attendant HPG axis suppression (usually manifested as a decreased serum testosterone among male participants) are generally associated with an energy deficit (33-35). Previously, we conducted a detailed assessment of energy balance in this cohort using doubly labelled water and weighted dietary analysis, finding that men had significantly higher energy expenditure and energy intake, and a more negative energy balance overall, compared with women (16). There were no significant sex interactions in basal serum androgen or cortisol levels over time. In the present cohort of women we also measured energy availability (energy intake minus exercise energy expenditure) (17), and while energy availability was low (range –10 ±11 to 23 ±15 kcal.kg FFM.d^{-1}, depending on the time of training and measure used), sex differences in energy deficit cannot plausibly account for the difference in cortisol levels we observe herein.

These data suggest that there are complex influences on HPG axis function and point to factors other than HPA axis function. There was a lack of clear sex-biased effect on LH and FSH during intensive military training. These findings support previous work in humans, demonstrating intact endocrine functionality in females exposed to

significant, sustained environmental and psychological stressors (36). While no statistical interaction was observed between the sexes over time, the sex difference in gonadotroph function was numerically smaller at week 29 than week 1. We previously reported suppressed gonadotroph responsiveness during training, among the larger cohort of women (also including COCP users) (14). These data indicate that physiological stress, likely associated with CRH and/or AVP stimulation, was greater among women, manifesting as relatively enhanced adrenal responsiveness after 29 weeks. A study with greater statistical power would be required to determine if a significant sex difference in gonadotroph function is seen over time.

Pituitary sensitivity to GnRH is influenced by upstream peptides including kisspeptin, and varies according to sex and across the phases of the menstrual cycle (37). Previously published urinary hormone data suggest the non-contraceptive users remained in the follicular phase throughout the study (14). There was also no difference in gonadotroph response between women using progestogen only contraception and non-contraceptive users. The greater gonadotroph response among women than men may reflect physiological differences in gonadotroph sensitivity. At the time of writing we are not aware of any published data which give sex-adjusted normative values for fold-wise response to a low dose GnRH test.

A possible explanation for the heightened HPA axis response among women lies in the neuroendocrine differences between sexes. Oestrogens have been implicated in enhancing HPA axis sensitivity (5, 6). Oestrogen receptors are abundantly expressed in the paraventricular nucleus which is central to HPA axis regulation and is closely associated with the suprachiasmatic nucleus — the circadian clock (38). Sleep deprivation is commonly reported during military training (39), and was marked during the study (40). Altered sleep has a potentiating effect on HPA axis reactivity (reviewed by Dalfsen and Markus (41)) and sex differences have been observed in HPA axis vulnerability to sleep disturbance. An enhanced HPA axis response to CRH was seen among women with poor sleep compared with a smaller cohort of men with poor sleep (42), while 8-year old (43) and adolescent girls (44) demonstrated an enhanced response to a standardised stress test following sleep deprivation compared with adolescent boys. The underlying reason for a greater response in women is not clear but could relate to increased oestrogen receptor-mediated sensitivity amplifying crosstalk between the paraventricular and suprachiasmatic

nuclei (38), or given findings from a range of ages and hormonal milieus, it seems plausible that some sex-associated effect of sleep disturbance on the HPA axis may exist independently of sex steroids.

Arginine vasopressin is an important hypothalamic activator of the HPA axis and demonstrates a striking sex dimorphism in rodents, with females showing greater expression of AVP following stress (38). Co-activation of AVP and the HPA axis was observed in women but not in men during an insulin stress test (45). Impaired hydration and heat stress are encountered frequently during military training and may have potentiated the stress response in women, although hydration status was not directly measured. Future studies should address the importance of sex dimorphisms in the co-activation of AVP and HPA axis in response to environmental stress.

Military training is complex and entails multifaceted psychological and social challenges as well as physical stressors, for example immersion in a nuanced sociocultural environment, reduced volition, simulated threats, and continuous assessment. We observed slight overall increases in scores of depression and anxiety, with no change in resilience. There was no effect of sex observed over time, however we did observe more adverse events in women than men. A larger study measuring ethnographic stress, coping and psychological wellbeing may elucidate sex differences in responses to multi-stressor training.

Discordant morning salivary and hair cortisol during training highlights the importance of sampling protocols in studies examining cortisol abundance in humans. While hair cortisol was similar between men and women during the first 3 months, it was higher among women thereafter; for morning salivary cortisol, the opposite was seen. Greater anticipatory stress drives morning cortisol (31), which may have been more frequent in women during the early stages of training, and in men during week 20 (the week of competitive regimental selection). However, overall cortisol exposure was similar in the early weeks of training, driven perhaps by the multiple, frequent stressors experienced at this stage — the 'shock of capture'.

Strengths of this study include the combination of dynamic and basal hormone markers over a long duration. We believe this is the first time that a low-dose pituitary function test has been applied or combined with basal cortisol markers during multi-stressor training. The multi-stressor exposure is relevant to the increasing diversity of gender

in arduous employment roles. Participants were well-matched in terms of age and demographics and these findings are supported by detailed measurement of energy balance reported elsewhere(16).

Limitations of this work include the sample size of men, caused by ethical constraints on recruitment, and relatively low numbers of women commencing the Commissioning Course. The training requirements meant that we were unable to synchronise tests to menstrual cycle (there was a 1-week window in which to arrange testing). There was significant variability in cycle timing during both tests, contributing to high variability in LH; however, by using fold-wise responses we were able to detect trends in pituitary responsiveness and make meaningful comparisons across groups. Moreover, due to course requirements, it was not possible to perform dynamic function testing in the early morning.

In conclusion, military training enhanced the HPA axis response and increased average cortisol exposure among women, but not men. This HPA axis response was associated with limited evidence suggesting a greater suppression of HPG axis response in women than men. These findings suggest that there are complex influences on HPG axis function, which extend beyond HPA axis activation. It is important to consider sex biased differences in strategies to address stressors, such as sleep deprivation, hydration status or psychological coping strategies.

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Figure Legends

Figure 1. Male and female gonadotrophin responses to 10µL gonadorelin during weeks 1 and 29 of military training. A 2-way ANOVA showed no significant sex × time interaction. Data are mean ± SEM.

Figure 2. Male and female cortisol response to 1µL ACTH-(1-24) over 1 hour during weeks 1 and 29 of military training. Significant sex × time interaction, for the area under the curve F 18.0, $p<0.001$. Data are mean ± SEM.

Figure 3. Hair and salivary cortisol. A: Natural logarithm of hair cortisol concentrations, B: Morning salivary cortisol concentrations, C: Evening salivary cortisol concentrations. Significant sex × time interactions were observed for hair cortisol ($p = .024$) and morning salivary cortisol ($p= .005$), but not evening salivary cortisol ($p = 0.3$). Week 14 saliva cortisol was excluded from statistical analysis due to missing data. Data are mean ± SEM. Ln: natural logarithm. * Significant sex difference observed, independent samples t-test $p<0.05$.