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Full title ***Reproductive and metabolic adaptation to multi-stressor training in women***

Short title ***Female endocrine adaptations to arduous training***

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Contributorship. RMG conceived and designed the study with supervisory input from JPG, RAA, DRW and RMR. RMG initiated the study with help from SLW, TJO and RLD in implementation. NZMH and AFH carried out technical analysis. RMG conducted the primary statistical analysis and drafted the manuscript with supervisory input from RAA, DRW and RMR. All authors contributed to refinement of the study protocol and approved the final manuscript

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Abstract

Hypothalamic-pituitary-gonadal (HPG) axis suppression in exercising women can be caused by low energy availability (EA) but the impact of a real-world, multi-stressor training environment on reproductive and metabolic function is unknown. This study aimed to characterize reproductive and metabolic adaptation in women undertaking basic military training. **Design:** Prospective cohort study in women undertaking 11-month initial military training (n=47). Dynamic low dose 1-hour GnRH tests were completed after 0 and 7 months of training. Urine progesterone was sampled weekly throughout. Body composition (dual x-ray absorptiometry), fasting insulin resistance (homeostatic modelling assessment 2, HOMA2), leptin, sex steroids, AMH and inhibin B were measured after 0, 7 and 11 months with an additional assessment of body composition at 3 months. **Results:** LH and FSH responses were suppressed after 7 months (both $p<0.001$). Among non-contraceptive users (n=20), 65% had regular (23–35d) cycles pre-enrolment, falling to 24% by 7 months of training. Of women in whom urine progesterone was measured (n=24), 87% of cycles showed no evidence of ovulation. There was little change in AMH, LH and estradiol, although inhibin B and FSH increased ($p<0.05$). Fat mass fluctuated during training but at month 11 was unchanged from baseline. Fat-free mass did not change. Visceral adiposity, HOMA2 and leptin increased (all $p<0.001$). **Conclusions:** HPG axis suppression with anovulation occurred in response to training without evidence of low EA. Increased insulin resistance may have contributed to the observed pituitary and ovarian dysfunction. Our findings are likely to represent an adaptive response of reproductive function to the multi-stressor nature of military training.

New and Noteworthy

Women commonly undertake arduous training across many types of employment and recreation. This is the first study to characterise detailed reproductive endocrine adaptation to prolonged arduous multi-stressor training in women.

We used a novel panel of dynamic and basal endocrine markers, urinary progesterone tracking and body composition over 11 months of compressed military training.

We identified marked suppression of hypothalamic-pituitary-gonad (HPG) axis function during training, but there was no evidence to suggest low energy availability despite high energy requirements.

The findings of this study suggest a complex interplay of psychological and environmental stressors led to suppression of the HPG axis via activation of the hypothalamic-pituitary adrenal (HPA) axis, due to non-exercise stressors.

Our findings develop add to extant understanding of female physiological endocrine adaptations to arduous training, suggesting that low energy availability might not be the only consideration to maintain reproductive health. The neuroendocrine impact of other stressors on the HPG axis like sleep restriction and externalised locus of control, which also upregulate HPA axis activity, should be considered.

Background

The female hypothalamic-pituitary-gonadal (HPG) axis is influenced by a variety of non-reproductive regulators. These include physiological adaptations to metabolic challenges which may be encountered through active occupations or lifestyles, including strenuous exercise, insufficient or excessive dietary energy intake, psychological stress and sleep disturbance (4, 21, 22).

Adaptive suppression of gonadotrophin releasing hormone (GnRH) drive is an important cause of hypothalamic amenorrhea in women undertaking high levels of physical activity, and has been ascribed to relative deficiency of dietary energy intake (low energy availability, EA) to the hypothalamus (52). Low EA decreases leptin and ghrelin, which mediate hypothalamic amenorrhea both through effects on GnRH secretion (24), and by upregulating the hypothalamic-pituitary-adrenal (HPA) axis (36). The HPA axis can also be activated by psychological stress (40) or sleep disturbance (48). Activation of the HPA axis modulates the HPG axis (49), however the 'stress' of exercise itself does not suppress the HPG axis when EA is sufficient (31). Conversely, overfeeding and adiposity are associated with disruption of HPG axis function relating to high insulin, androgen and estradiol concentrations (6).

Women are increasingly undertaking physically demanding and stressful employment, but the contribution of low EA and non-EA stressors to HPG axis dysfunction in these contexts is not known. Military training provides a valuable model of heavy exercise amongst other stressors, e.g. sleep disruption, externalized locus of control and ethnographic challenges (20), which are commonly encountered in a variety of other occupations and lifestyles. To be eligible for such training, women are of reproductive age (13) and their hormonal milieu is commonly altered by use of synthetic estrogen or progestin-containing contraceptives (14).

The Female Endocrinology in Arduous Training (FEAT) Study examined an arduous basic combat training program lasting 11 months in a cohort of women undertaking the Commissioning Course at the Royal Military Academy, Sandhurst, UK. We have previously

described HPA axis activity (19) the determination of EA (16) and skeletal adaptations (38) among participants from the FEAT Study. The findings highlighted the arduous nature of training, including energy demands of up to 3,800 kcal/d (of which up to 2,600kcal/d was measured as physical activity), working more than 16h per day (16); declining mood and resilience, increased fatigue (19) and short duration of sleep (29). These factors were proposed to contribute to the observed increases in dynamic cortisol response and average cortisol concentrations (19).

Despite an intensive protocol which included gold-standard assessments energy requirement, we were unable to measure EA precisely, due to under-reporting of energy intake (16). Underestimation of reported dietary intake is well-recognized as a challenge to EA measurement in the field (7, 8) and has led researchers to identify surrogates of low EA, such as hormonal markers including decreased leptin, insulin, free and total triiodothyronine (T3), insulin-like growth factor 1 (IGF-1) and estradiol, and increased fasting cortisol (12). Such measures are promising alternative to direct measurements of EA in the field.

The primary aim of this analysis was to examine reproductive function (including menstrual function, ovulation, and basal and dynamic reproductive hormones), over time in non-contraceptive users, alongside biochemical surrogates of metabolic status and body composition (long-term changes reflecting energy balance). The secondary aim was to assess changes in these parameters among women whose HPG axis was under continuous negative feedback from systemic hormonal contraceptive use (menstrual function and ovulation were excluded from the secondary analysis). We hypothesized that evidence of hypothalamic amenorrhea (in non-contraceptive users) and reduced pituitary gonadotroph response (in all women) would be observed in association with evidence of low EA.

Methods

Overview

This prospective cohort study observed reproductive and metabolic effects on women of undertaking the 11-month Commissioning Course. The study was designed to fit around the compressed training program and comprised dynamic endocrine function tests, fasting blood sampling, dual-energy x-ray absorptiometry (DXA) body composition scans, and continuous, longitudinal tracking of ovulation and menstrual function (**Figure 1**).

The Commissioning Course

This residential infantry-based course trains mixed sex groups of Officer Cadets in leadership over three 14-week terms. Participants are usually naïve to military life beforehand. Components include field exercises (simulating austere deployments lasting 3 days to 4 weeks), academic study and rigorous physical activity. Programmed activities last from early morning until late at night and include many weekends. The first two terms are particularly arduous, comprising initiation into the Army followed competitive selection for Army regiments. The focus of the final term is more academic and culminates in commissioning to become Army Officers. Officer Cadets consume either carbohydrate-rich canteen food or field rations, frequently supplemented with high calorie snacks (16).

Participants

Women enrolling in the Commissioning Course between February 2017 and January 2018 were invited to participate in the study across nine pre-Course briefings. The number of participants was limited to women enrolling on the Course (5 to 15 per briefing) and the duration of the study (limited to 2 years by Course directors), and thus represents a convenience sample. Inclusion criteria were aged 18-30 years and female sex. Exclusion criteria were pregnancy, known history of adrenal, ovarian or GnRH insufficiency, pituitary disease, thyroid disease in the past year, diabetes, hyperparathyroidism, osteopenia, oral, inhaled or topical glucocorticoid use or ongoing musculoskeletal injury. All participants provided written informed consent. Ethical approval was obtained from the Ministry of

Defence Research Committee (790/MoDREC/16). The study was conducted in accordance with the Declaration of Helsinki.

Baseline assessment and questionnaire

At study baseline, information including alcohol consumption, smoking and a comprehensive reproductive and medical history were recorded, including indication and type of any hormonal contraception used, or menstrual history if not using hormonal contraception, and age of menarche. Height and weight were assessed semi-nude (wearing t-shirt and shorts) using a stadiometer and scales (Seca models 213 and 874, respectively, Seca, Birmingham, UK).

Menstrual function and ovulation

Participants not using hormonal contraceptives completed a menstrual diary throughout the study. Incomplete diary data (e.g., due to diaries being lost), were addressed using retrospective recall at the end of each term to record date of last menstrual period, menstrual period number and regularity, as well as current contraception use. Cycle length definitions used were eumenorrheic: 26–35 days, oligomenorrheic: 36–89 days, and amenorrheic ≥90 days.

Ovulation was detected in non-contraceptive users and in IUS users using changes in progesterone: creatinine ratio from serial urine aliquots collected on the same day of each week (17). Daily samples were collected for the first 30 days of training only, aiming to describe a complete or nearly complete baseline cycle for that individual (index cycle). Only days 7, 14, 21 and 28 from the index cycle were used for statistical analysis, but all daily samples were used to identify the baseline luteal phase duration and to corroborate the identification of subsequent suggested luteal phase defects (LPD) (examples shown in **Supplementary figure 1**, https://figshare.com/s/abf86ed26a6d99455987). A longer duration of daily sampling was not possible due to the constraints of compressed military training. Ovulation was identified from our previously defined threshold of 11.51 pmol/mmol (daily

samples from ovulatory women) (17). Suggested LPD was defined as a 2-fold rise in progesterone: creatinine ratio from the follicular phase or urine progesterone concentration between 6.36 pmol/mmol and 11.50 pmol/mmol, and not luteal phase duration (only assessed during index cycle). Urinary progesterone concentrations which did not reach a threshold concentration of a 2-fold rise from follicular to luteal phases *and* were ≤6.36 pmol/mmol were defined as showing no evidence of ovulation.

Dynamic GnRH test

A low-dose GnRH (Gonadorelin ®) test was used to identify changes in pituitary function at week 1 of term 1 and the final week of term 2 (37). Due to constraints imposed by the training schedule, GnRH testing was completed in the late afternoon. Participants were allowed to relax before a 20G cannula (B Braun, Dublin, Ireland) was inserted into an antecubital fossa vein. A sample of blood was taken from the cannula in ethylenediaminetetraacetic acid (EDTA)-containing tubes. After 10-15 minutes, 10µg gonadorelin hydrochloride (Intrapharm, Maidenhead, UK) was injected followed by a 10mL saline flush. Venous blood was sampled from the cannula in EDTA-containing tubes after 20, 30, 40 and 60 minutes.

Body composition, fasting blood sampling

At baseline and at the ends of terms 1, 2 and 3, body composition was assessed using dual energy x-ray absorptiometry (DXA) (GE Lunar iDXA, GE Healthcare, Chalfont St Giles, UK). Whole body regional lean and fat mass were computed using enCORE Body Composition software (GE Healthcare). Predefined regions were arms, legs, trunk, android (area between the ribs and pelvis) and gynoid (pelvis and upper thighs). An additional module (CoreScan ®, GE Healthcare) assessed mass and volume of visceral adipose tissue within the android region. In the first week of term 1 and final weeks of terms 2 and 3, at around 0800 h, after fasting from 2200 h, a venous blood sample was drawn into EDTA, serum-separating gel and fluoride oxalate tubes (Monovette®, Sarstedt, Nümbrecht, Germany).

Laboratory methods

Serum and plasma were stored at −80°C until analysis. Plasma luteinizing hormone (LH), follicle-stimulating hormone (FSH), estradiol, and progesterone, and urine creatinine and progesterone were assayed by Abbott Architect ® (Abbot, Longford, Ireland) according to manufacturer's instructions (17). Leptin and inhibin B were measured from plasma by ELISA (Quantikine, USA and Beckman Coulter, High Wycombe, UK, respectively). IGF-1, insulin, C-peptide, thyroid stimulating hormone (TSH), free thyroxine (T4), total T3, anti-Müllerian hormone (AMH), prolactin and sex hormone binding globulin (SHBG) were measured from gel-separated serum using a Roche Cobas e411 analyzer (Roche Diagnostics GmbH, Mannheim, Germany). Glucose was determined from plasma containing fluoride oxalate and non-esterified fatty acids (NEFA) from serum using commercial kits (Alpha Laboratories, Eastleigh, UK) adapted for use on a Cobas Fara centrifugal analyzer (Roche, UK). Inter-assay coefficient of variation (CV) was <4% for Architect ®, e411, Fara assays and intra-assay CV was <10% for all ELISAs. Intra- and inter-batch CVs were <2.5% for Architect assays (urine analyses were completed in batches over several days).

Dihydroepiandrostenedione (DHEA), androstenedione, 17-OH progesterone and testosterone were quantified simultaneously in plasma (200 uL) by liquid chromatography mass spectrometry. A calibration curve for these steroids was prepared alongside plasma samples (200 µL) enriched with isotopically labelled analytes. 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 (45)). Chromatographic separation was achieved following injection (20 µL) using a gradient on a Shimadzu Nexera UHPLC system on a Kinetex C18 (150 x 3 mm; 2 µm) column of mobile phases: 0.1 % formic acid (FA) in water, 0.1 % FA in methanol, 0.5 mL/min, 30 °C, followed by Mass spectrometry (MS)/MS analysis on a Sciex QTrap 6500+ operated in positive ESI, where MS settings have been described previously (47). 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).

Statistical analyses

Participant description at baseline. Data are expressed as mean (standard deviation, SD) for normally distributed variables and median (IQR, interquartile range) for other data. The Homeostatic Model Assessment of insulin resistance 2 (HOMA2) was calculated using the Oxford Centre for Diabetes Endocrinology and Metabolism Diabetes Trials Unit calculator (https://www.dtu.ox.ac.uk/homacalculator/). Non-normal data were log transformed (LH, FSH and their ratio, leptin, inhibin B, estradiol, progesterone, SHBG, free androgen index, insulin, AMH) and normal distribution of transformed data was confirmed prior to analysis with parametric statistics. Participants were categorized into groups depending on hormonal contraception use due to the known effects of high concentrations of sex steroids on HPG axis central negative feedback: combined oral contraceptive pill (COCP, n=13), progestogen-only contraception (POC, e.g., progestogen-only pill, progestogen-eluting implant or intramuscular medroxyprogesterone, n=16) and no hormonal contraceptive (Nil, n=14) or intrauterine system (IUS, n=4, Mirena ® or Jaydess ®). The Nil and IUS group were pooled (n=18) since IUS delivers progestogen locally and does not impact the HPG axis centrally or interrupt ovulation (3), confirmed by a comparison of relevant parameters in Nil and IUS using independent samples t-tests (**Supplementary Table 1**, https://figshare.com/s/abf86ed26a6d99455987). Participants who changed hormonal contraception between tests (n=5) were excluded from subsequent analysis. Two of the four women who commenced COCP during the study recorded menstrual function for 3 and 4 months, respectively. Four women who withdrew from the study recorded menstrual function beforehand and for 3, 3, 6 and 8 months, respectively. Menstrual cycle data were not analyzed for the four women who used an IUS because of the local effects of levonorgestrel, resulting in erratic or infrequent (two women) or absent menses (two women). Participants

who withdrew were compared at baseline with those who completed the study using Chi-square (binomial variables) or independent samples t-test (continuous variables).

Assessment of ovulation and menstrual status. Menstrual periods were plotted against time for non-contraceptive users and urine progesterone concentrations for Nil/IUS. For each term, participants were categorized as ovulatory if two or more ovulatory cycles were detected from weekly samples, suggested LPD if two or more cycles met LPD criteria (17), and no evidence of ovulation if fewer than two cycles met ovulatory or LPD criteria. Ovulation and menstrual status were compared across terms using Chi squared tests.

Changes in dynamic responses to GnRH. Fold difference in LH and FSH from immediately before GnRH administration was calculated to provide maximum fold increase and area under the curve of 1-h fold response to GnRH (AUC, calculated by the trapezoidal rule). Fold-wise change was used to account for variation in baseline LH and FSH (timing of tests could not be controlled for cycle day). The primary analysis, change in the Nil/IUS group from visit 1 to visit 3 was calculated using paired samples t-tests. The secondary analysis, change contraceptive group $\times$ time, was performed using a two-way ANOVA with Bonferroni *post-hoc* tests for significant main effects and interactions.

Changes in fasting blood markers and body composition. The primary analysis, change over time in the Nil/IUS group, was analyzed by repeated measures ANOVA. The secondary analysis, contraceptive group $\times$ time, was analyzed using a mixed-design repeated measures ANOVA, with effect sizes reported as partial Eta squared (η_p^2). Where a significant main effect or interactions were identified, baseline was compared with subsequent measurements using paired sample t-tests.

Statistical analysis was performed using SPSS version 24.0 for Macintosh (IBM, Armonk, NY, USA). Significance was accepted as $p<0.05$ with Bonferroni adjustment for multiple comparisons (adjusted p values are presented alongside comparisons).

Results

Participants, baseline assessment

Of 77 women who attended the study briefing and were eligible, 68 (88%) volunteered to participate (**Supplementary Figure 2**, https://figshare.com/s/abf86ed26a6d99455987). Of these initial volunteers, seven (10%) did not participate in the study or undergo baseline assessment. Nine participants (15%) withdrew after baseline assessment due to injury, declining to participate or voluntary resignation from the Army. The contraceptive-taking groups comprised 13 participants who used a COCP, 16 who used POC (9 progestogen-eluting contraceptive implant, 6 progestogen-only pill and 1 intramuscular medroxyprogesterone acetate), 14 nonusers and 4 who used an IUS. A further five (8%) were excluded because they changed contraceptive method during the study. A complete data set is presented on 47 women.

The physical characteristics of participants in contraceptive groups did not differ. There were no differences in demographic, anthropometric, reproductive or lifestyle factors, or in nutritional or reproductive markers between women who completed the study and those who withdrew (**Supplementary Table 2**, https://figshare.com/s/abf86ed26a6d99455987). Fasted TSH, free T4, prolactin, LH, FSH, LH:FSH ratio, androstenedione, free and total testosterone, SHBG, DHEA, 17-OH progesterone and creatinine were within normal limits in all participants prior to participation (**Supplementary Table 2**, https://figshare.com/s/abf86ed26a6d99455987).

Menstrual function and ovulation

Changes in menstrual function are shown **Figure 2 A and B**. Of 20 women who recorded menstrual function, five (25%), 11 (65%) and six (43%) were oligo/amenorrheic in terms 1, 2 and 3, respectively. Menstrual function did not differ significantly between terms (χ^2 23.4, p=0.32).

During the baseline index cycle characterized from daily urine samples in 24 women, only 3 (13%) were deemed ovulatory, with suggested LPD in 7 (25%) and 14 (50%) showed no evidence of ovulation. Median luteal phase duration was 9 (IQR 6 to 11) days. (examples are shown in **Supplementary Figure 1**, https://figshare.com/s/abf86ed26a6d99455987). Ovulatory function from weekly samples is shown in **Figure 2 C and D.** The ovulatory urinary progesterone threshold of 11.51 pmol/mmol was achieved in 25 cycles (16%) in 12 women. In terms 1, 2 and 3, respectively three (13%), one (5%) and two (17%) participants were ovulatory. The prevalence of ovulation varied significantly between terms (χ^2 18.5, p=0.005), being lowest during term 2.

Dynamic and fasting HPG axis markers

Dynamic HPG axis markers are shown in **Figure 3**. No significant difference was seen in maximal or AUC fold-change response in LH or FSH in Nil/IUS. However, a significant decrease in both LH and FSH responses to GnRH was seen across contraceptive groups (significant effect of time, no effect of group $\times$ time).

Fasting markers of HPG axis function were unchanged in Nil/IUS (main markers of interest shown in **Figure 4,** others shown in **Supplementary Table 3**, https://figshare.com/s/abf86ed26a6d99455987)). When contraceptive groups were pooled in the secondary analysis, increased inhibin B and FSH were observed throughout, and there was an upward trend in estradiol in Nil/IUS and POC groups (significant main effect of time, but no effect of group $\times$ time, p=0.085). Free androgen index and inhibin B were significantly lower among COCP users than other groups however there was no change in androgen concentrations. SHBG increased modestly in POC and COCP groups by week 29 before decreasing to baseline by the end of the study.

Body composition

Total body mass did not change significantly over time in Nil/IUS, although there was a significant mean (SD) 1.1 (0.5) kg loss in fat mass by week 14 followed by a 1.8 (0.8) kg gain by week 29, but by week 43, fat mass was identical to baseline; p=0.001 (Table 1). No significant differences were seen between groups in body mass, except for fat-free mass where modestly greater gains throughout training were seen in Nil/IUS than other groups (ηp^2 0.150, p=0.002; **Supplementary Table 4**, https://figshare.com/s/abf86ed26a6d99455987). When groups were pooled, significant changes were seen in total and fat mass, following the same trend as Nil/IUS, with small effect sizes **(Table 1, Supplementary Table 4**, https://figshare.com/s/abf86ed26a6d99455987). Regional changes demonstrated loss, followed by gain, of fat in the android region, and to a lesser extent in the trunk. Visceral adipose volume and mass increased modestly at every visit (total increase of 35.9 ± 74.0 g, p=0.008; no effect between groups).

Fasting metabolic markers

Leptin and HOMA2 increased significantly while NEFA decreased significantly in NIL/ IUS during training. There was a modest but significant increase in IGF-1 during training. There was no change in total T3, while modest but significant rises were seen in TSH and free T4 (**Figure 4**). Changes in all participants reflected those seen in Nil/ IUS, with increases in leptin, HOMA2, glucose and TSH, and decreases in NEFA and free T4, with no change in total T3. No change in IGF-1 was seen in the analysis of all participants. There were no interactions of group × time for fasting metabolic markers (**Figure 4**). TSH and thus total T3 were increased significantly among non-COCP users only.

Discussion

We undertook a detailed assessment of changes in HPG axis function in women during military training lasting 11 months. When considered in the context of our assessment of EA in this study (16), the fasting metabolic measures presented here (particularly increased leptin and IGF-1, and unchanged estradiol and total T3) support the view that EA was sufficient overall. Our *a priori* anticipation was that, without a substantial energy deficit or evidence of low EA, the training would not induce reproductive dysfunction. However, there was strikingly little evidence of ovulation in the Nil/IUS group, and gonadotroph responsiveness was suppressed across all contraceptive users after 29 weeks. Therefore, our hypothesis that hypothalamic amenorrhea and HPG axis suppression would be associated with evidence of low EA was rejected. We have reported elsewhere that activity of the HPA axis was upregulated due to stressors like sleep deprivation and externalized locus of control (19), and we consider that such stressors could have contributed to hypothalamic amenorrhea and HPG axis suppression in this population, independent of EA.

The high rates of menstrual disturbance observed in Nil/IUS were commensurate with other studies of military training (9, 20, 41). The paucity of ovulation and short luteal phases in the month of daily sampling suggest HPG axis disturbance occurred from the onset. Hypothalamic amenorrhea is a consequence of numerous neuromodulatory signal changes, reduced GnRH pulsatility and amplitude and suppression of the HPG axis (21), though downstream pituitary gonadotroph effects are less clear.

While no changes were identified in LH and FSH response to GnRH in Nil/IUS, when considered alongside changes in women using the same contraceptives at each test (inducing a fixed negative feedback on the HPG axis), statistical power was sufficient to suggest a significant decrease in responses after 28 weeks across groups, the effect on LH being proportionately greater than FSH. This lends credence to the use of repeated GnRH tests to test changes in pituitary gonadotroph function in studies of contraceptive users; as

far as we are aware, ours is the first to do so. The validity of our finding is preliminary and based on the premise that feedback on the axis caused by hormonal contraception was the same within individuals at both timepoints, supported by consistent changes in SHBG, estradiol and progesterone commensurate with regular use.

Our observations are consistent with LH secretion being more dependent than FSH on GnRH pulsatile activity, as is well established (50). Others have found low EA-associated hypothalamic amenorrhea is associated with suppressed LH, FSH (to a lesser degree) and estradiol, which correspond with our observations (26, 39, 44). A study of patients with hypothalamic amenorrhea due to dieting, stress or an eating disorder demonstrated decreased LH, but unchanged FSH response to a 50μg GnRH test compared with the same patients after recovery of menses (43). However a cross-sectional study of athletic women with hypothalamic amenorrhea found the opposite: increased LH but blunted FSH response to 10μg GnRH, compared with eumenorrheic controls (33).

Inhibin B and AMH are clinically useful markers of ovarian function. The increases in inhibin B and possibly estradiol might suggest disproportional development of mid-sized to larger follicles in all contraceptive groups (30), likely driven by increased basal FSH (34). The growth of smaller, pre-ovulatory follicles, which are less gonadotropin dependent, appeared to continue unchanged as evidenced by stable levels of AMH. In a study of athletes with low EA, De Souza et al. found basal FSH and estradiol concentrations in the luteal-follicular transition were suppressed (11). Since FSH is strongly negatively determined by inhibin B in the absence of high estradiol concentrations (42), the observations of De Souza et al. may be consistent with ours. Since few if any studies have used the GnRH test in contraceptive users before, findings from these groups should be interpreted with caution.

The Course was highly physically demanding; average energy expenditure was over 4000 kcal/d, driven by long durations of moderate and vigorous physical activity (16). Owing to the time pressures of the training it was not possible to capture all energy intake to directly

measure EA (16). Energy balance, reflected in body compositional change, was unchanged from baseline by the end of the study, although fat and lean mass fluctuated in the intervening 11 months. Fat mass losses and lean mass gains were greatest during term 1, as was observed during 9-week US Army Basic Combat Training (15). The initial training focused on building basic military skills and fitness and Nil/ IUS showed the lowest proportion of ovulatory cycles. In the second term, when the more arduous combat exercises took place, body fat increased. These changes were associated with increases in leptin, glucose and HOMA2, which would not normally be observed in low EA (2, 12). Total T3, IGF-1 and estradiol, which are normally sensitive markers of low EA (12, 32), were unchanged across all participants. IGF-1 increased significantly in Nil/ IUS, in contrast to the decrease that is normally observed in low EA (12). NEFA concentrations reflect insulin resistance in obese individuals (27), but the progressive decrease we observed may have been a response of adipose tissue to increasing insulin levels and possibly high levels of physical activity (25). Increased TSH and decreased free T4 indicated subtle reductions in thyroid gland activity and may reflect known effects of increasing estrogens on the thyroid axis (51), an interpretation supported by the differences observed between contraceptive groups in TSH.

Multiple stressors were characterised during the FEAT Study including high energy demands and long working hours (16), psychological stress and fatigue (19) and short sleep duration (29). The HPA axis was upregulated during training, however putatively beneficial adaptations were observed in autonomic function and physical fitness (19) and skeletal remodeling (38). While the stress of exercising *per se* does not induce the metabolic circumstances to suppress HPG axis function (23), it appears likely that stressful non-exercise factors explain our observations. Suppression of HPG axis function may be explained by stress-related increases in adrenal cortex responsiveness and average cortisol (1), sleep deprivation (28, 46), intermittent over-feeding following periods of negative energy balance (‘energy compensation’) (35) or a combination of these. Such a ‘multi-stressor’

etiology is a more plausible explanation than solely low EA; effects of low EA are temporary energy-conserving adaptations and reproductive function is restored when EA is restored (10). The more protracted HPG axis suppression we observed suggests that other stressors led to HPA axis activation, HPG axis suppression and hypothalamic amenorrhea evolution in a way that is more complex and nuanced than the cause-effect paradigm of low EA (23). For example, if it is not perceived as pleasurable or voluntary, exercise could be hypothesised to contribute to the psychological stress load in this multi-stressor model.

Strengths of our study included combining dynamic and basal testing of reproductive function over a long duration. It was pertinent to include contraceptive users to allow generalizability to women of reproductive age likely to undertake such training (5). However, the long-term impact of HPG axis suppression in addition to hormonal contraceptive use is unclear. Measuring EA in practice is challenging; our biochemical measures provided meaningful corroborative evidence of metabolic status, and multiple basal metabolic and reproductive measures were likely to improve sensitivity and specificity for low EA (12). Weekly urinary progesterone analysis suggested LPD and anovulation which would have been missed from menstrual function alone, and is a promising technique for field studies in future. The GnRH test has proved an effective field study measure of HPG axis function (18) allowing study visits to be organized around training schedules, not the reproductive cycle.

Limitations included the small sample size, in particular of non-hormonal contraceptive users, although as far as we are aware this is the largest study detailing reproductive function in women during arduous training. We could only include participants of European ancestry, which limits generalizability. The measurement of EA is difficult due to inaccuracy in energy intake measurement (8), hence we have relied on fasting biomarkers. The study was limited to indirect measures of ovulatory function, since it was not deemed feasible to undertake regular transvaginal ultrasound during training. It is possible that the index month did not capture a complete cycle, however further daily sampling was not possible at baseline due to the training program.

In conclusion, arduous multi-stressor training in women was associated evidence of hypothalamic amenorrhea, pituitary gonadotroph suppression and ovarian dysregulation, with metabolic maladaptation. Suppression of the HPG axis may have been compounded by other elements of training via upregulation of the HPA axis, including sleep restriction. Our findings represent a more complex evolution of reproductive dysfunction than low EA alone. It is unlikely that any single factor was responsible for the HPG axis suppression we observed, and a combination of stressors including exercise, psychological stress, energy compensation, and sleep restriction were putatively responsible. Focused studies are now required to explore the mechanisms underlying these observations.

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Table

	Baseline	Week 14	Week 29	Week 43	Time (main effect)			Group × time interaction	
					ηp^2	p (1)	p (2)	ηp^2	p (3)
Age, y Nil/ IUS All	 23.9 (2.6) 24.1 (2.6)								
Body mass index, kg.m^2 Nil/ IUS All	 23.6 (2.2) 23.3 ±2.1								
Total Mass, kg (all) Nil/ IUS All	 64.8 ±6.5 64.1 ±7.1	 64.8 ±6.6 63.6 ±7.2	 65.7 ±6.6 64.7 ±6.8	 65.5 ±6.4 64.3 ±6.9	0.134	0.089	0.006*	0.094	0.13
Fat mass, kg (all) Nil/ IUS All	 16 ±3.5 15.6 ±3.8	 15.1 ±3 14.5 ±3.4	 16.8 ±3 16.2 ±3.2	 16.2 ±3 15.6 ±3.3	0.321	0.001*	<0.0001*	0.255	0.99
Fat-free mass, kg (all) Nil/ IUS All	 48.7 ±5.3 48.5 ±5.3	 49.7 ±5 49.1 ±5.1	 48.9 ±5 48.5 ±4.9	 49.3 ±4.6 48.7 ±4.9	0.415	0.105	0.002*	0.105	0.001*

Table 1. Physical characteristics. (1) the primary analysis, changes in Nil/ IUS group over time (n=18); (2) changes in all participants (n=47); (3) interaction of contraceptive group × time. Data are mean ± SD. ηp^2 partial eta squared. * significant (p<0.008) after Bonferroni adjustment. Sub-group data are shown in supplementary table 4.

Figures

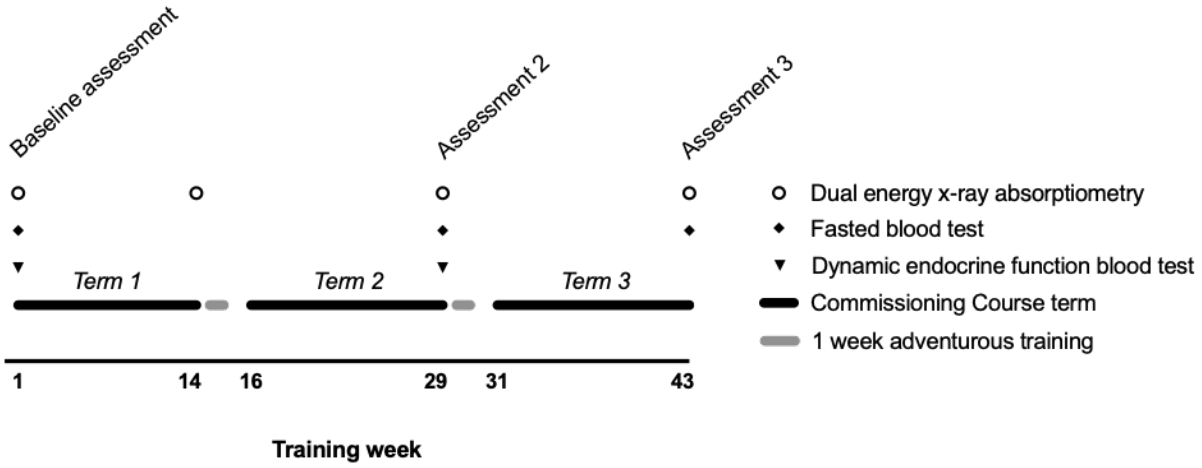

Figure 1 Study design

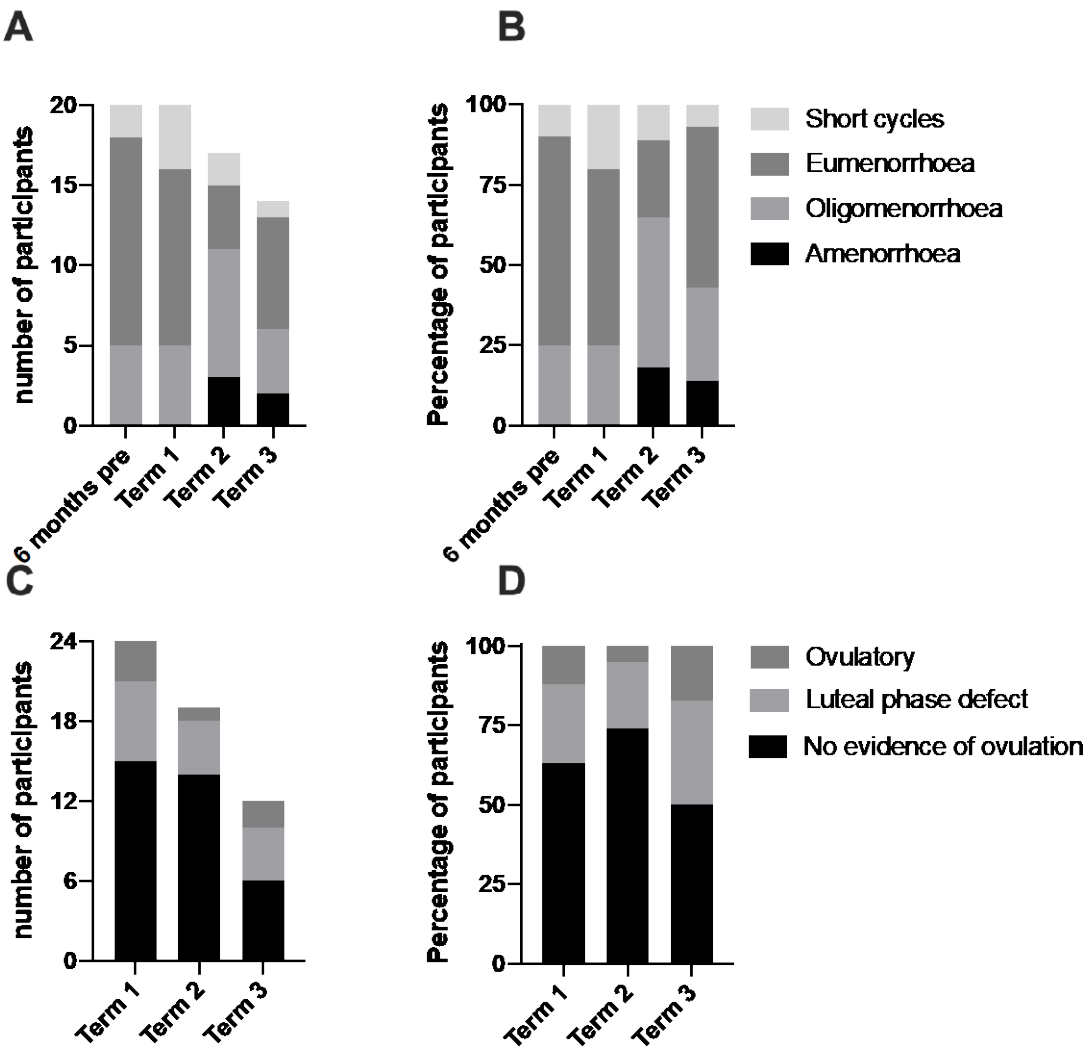

Figure 2. Menstrual and ovulatory function. Available data from Nil/ IUS (n=18) plus non-contraceptive using participants who dropped out of the study (n=6). Panels A and B: menstrual function; C and D: ovulatory function. LPD: luteal phase defect (definitions are provided in main text). Nil/ IUS: intrauterine system or non-contraception users (n=18).

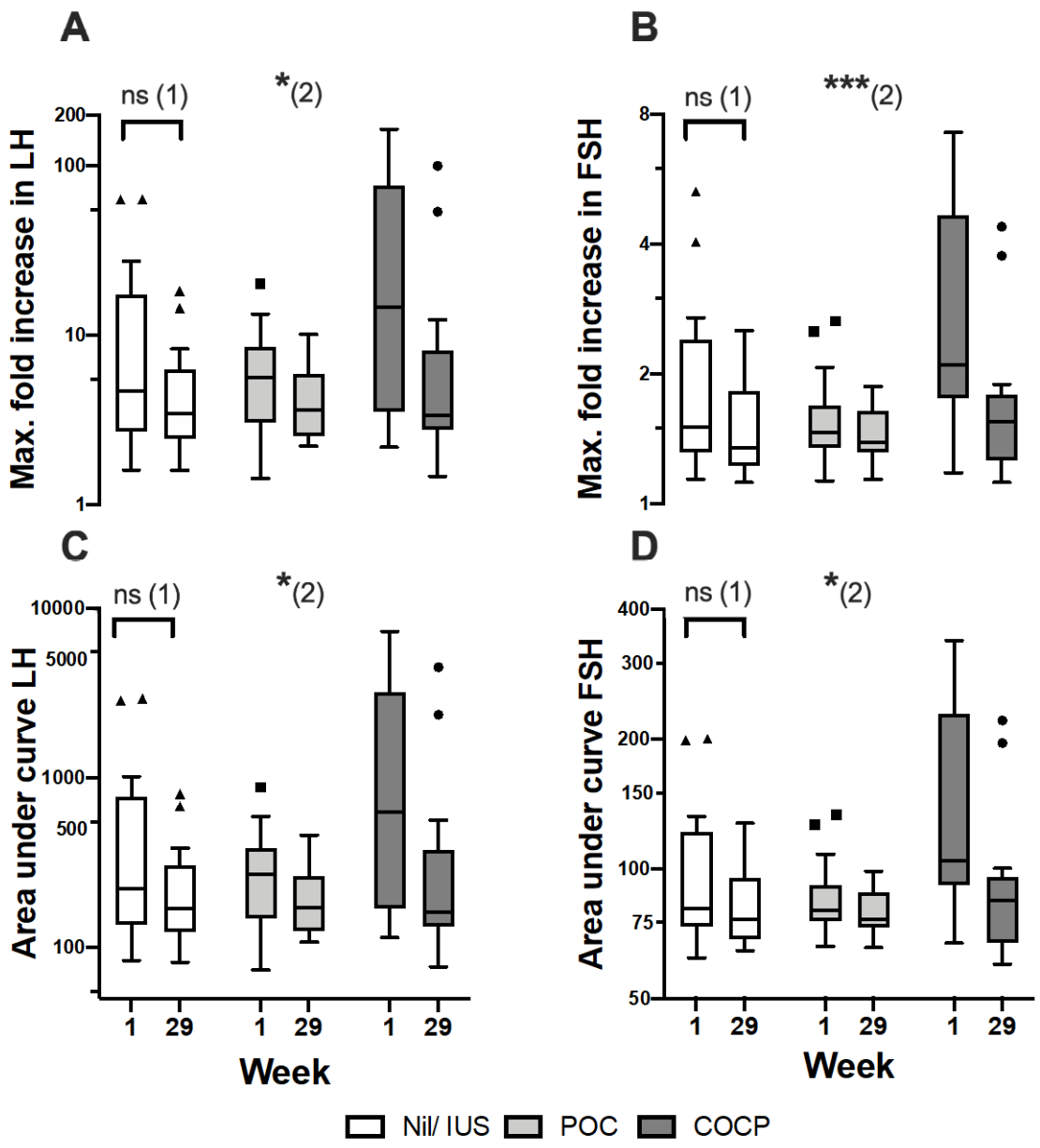

Figure 3. Response to GnRH test at week 1 and 29 of training. Panels A and B show maximal fold differences in luteinizing hormone (LH) and follicle stimulating hormone (FSH), respectively. Panels C and D show area under the curve of the fold-wise response over 1 hour in LH and FSH, respectively. (1) refers to the primary analysis, change in Nil/IUS. (2) main effect of time for all groups. There was no interaction of group □ time for response to GnRH tests. Due to the range of responses between groups, y axes are shown as a log scale. COCP: combined oral contraceptive pill users (n=13), POC: progesterone only

contraception users (n=16), Nil / IUS: intrauterine system or non-contraception users (n=18). ns: not significant * p<0.013 vs week 1 after Bonferroni adjustment, *** p<0.0001 vs week 1.

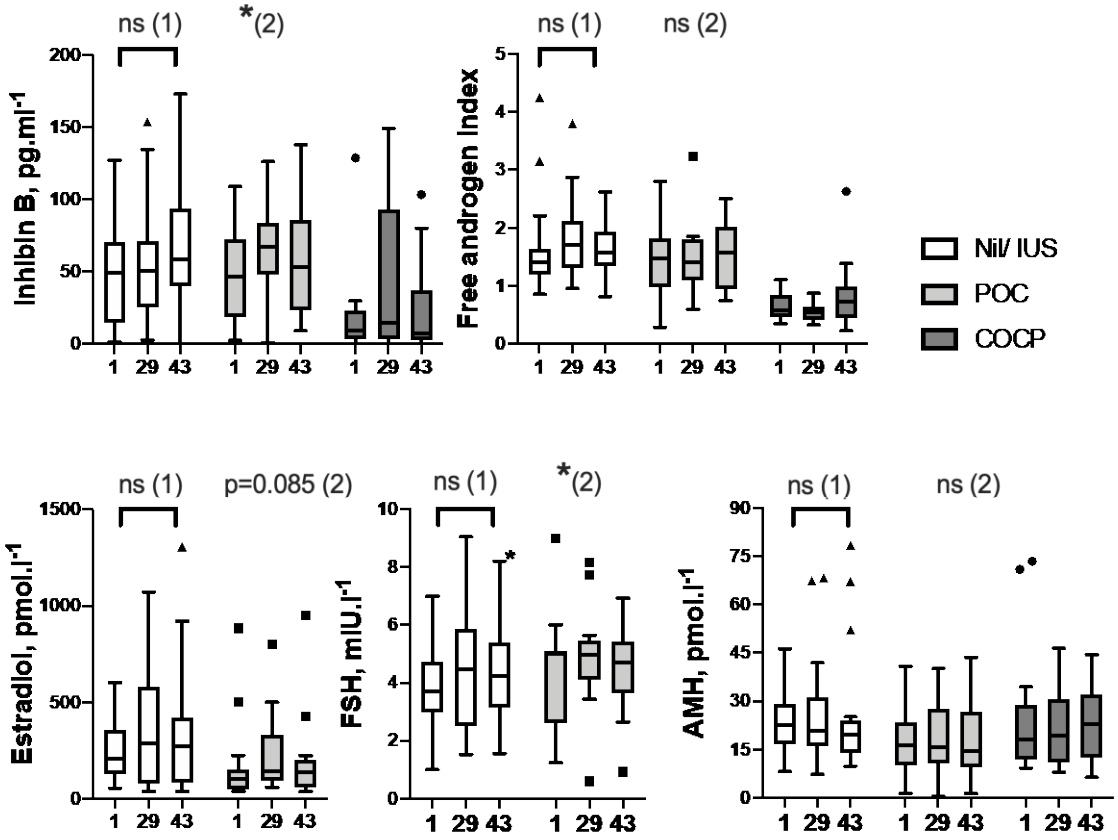

Figure 4. Basal markers of HPG axis function. (1) refers to primary analysis, change over time in Nil/ IUS. (2) refers to all participants, main effect of time. There was no significant interaction of group × time for any basal marker of HPG axis function. * p<0.01: significant after Bonferroni adjustment. COCP users were excluded from estradiol and FSH analyses due to direct suppressive effect of synthetic estrogen on basal levels of these parameters. COCP: combined oral contraceptive pill users (n=13), POC: progesterone only contraception users (n=16), Nil / IUS: intrauterine system or non-contraception users (n=18), AMH: anti-Müllerian hormone, FSH: follicle stimulating hormone

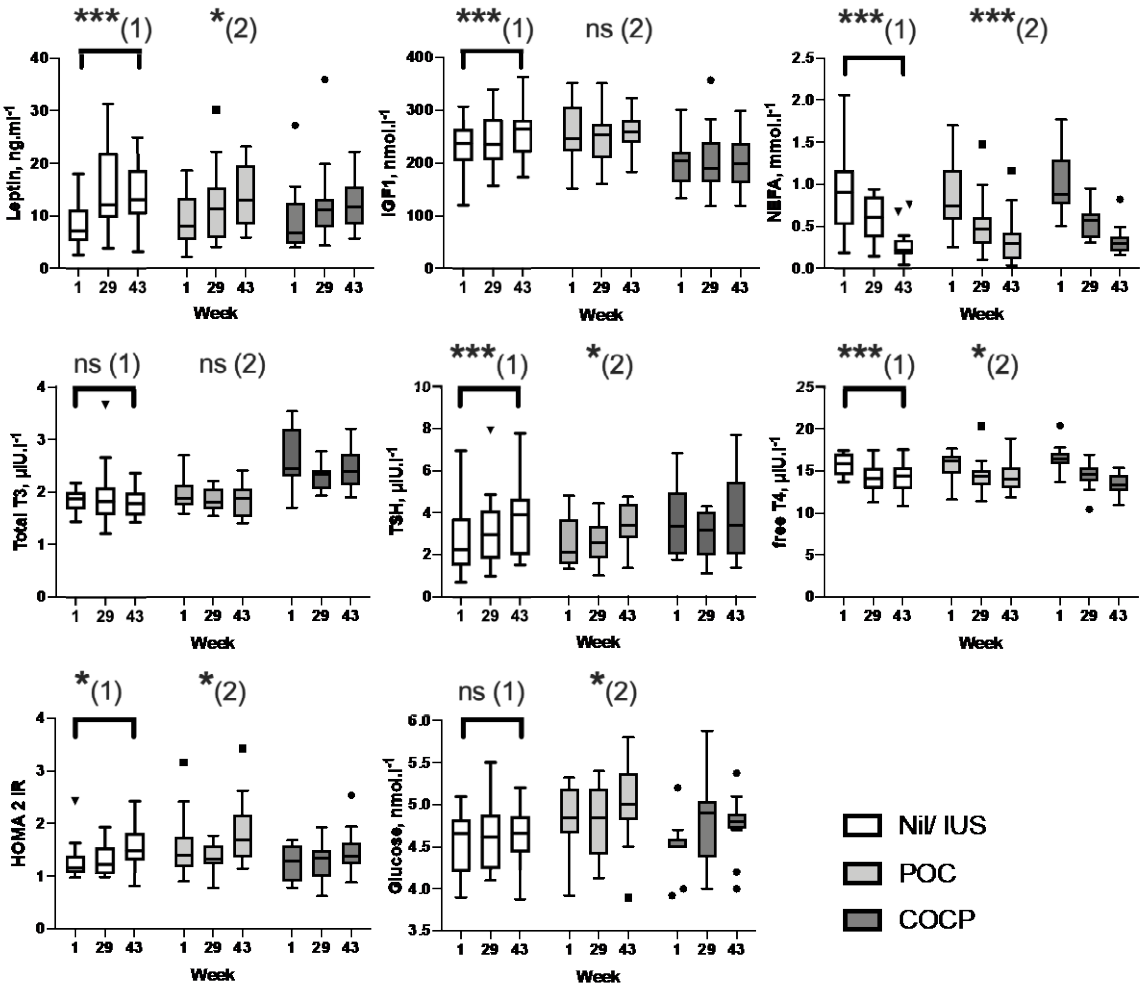

Figure 5. Fasting metabolic markers. (1) refers to primary analysis, change over time in Nil/ IUS. (2) all participants, main effect of time. There was no significant interaction of group × time for fasting metabolic markers. * p<0.006, *** p<0.0001, ns: not significant, after Bonferroni adjustment. IGF1: insulin-like growth factor 1, NEFA: non-esterified fatty acids, T3: triiodothyronine, TSH: thyroid stimulating hormone, T4: thyroxine, HOMA2 IR: homeostatic modelling assessment of insulin resistance 2. COCP: combined oral contraceptive pill users (n=13), POP: progesterone only contraception users (n=16), Nil / IUS: intrauterine system or non-contraception users (n=18).