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Luteinizing Hormone Pulsatility Is Disrupted at a Threshold of Energy Availability in Regularly Menstruating Women

ANNE B. LOUCKS AND JEAN R. THUMA

Department of Biological Sciences, Ohio University, Athens, Ohio 45701

To investigate the dependence of LH pulsatility on energy availability (dietary energy intake minus exercise energy expenditure), we measured LH pulsatility after manipulating the energy availability of 29 regularly menstruating, habitually sedentary, young women of normal body composition for 5 d in the early follicular phase. Subjects expended 15 kcal/kg of lean body mass (LBM) per day in supervised exercise at 70% of aerobic capacity while consuming a clinical dietary product to set energy availability at 45 and either 10, 20, or 30 kcal/kg LBM·d in two randomized trials separated by at least 2 months. Blood was sampled daily during treatments and at 10-min intervals for the next 24 h. Samples were assayed for LH, FSH, estradiol (E_2), glucose, β-hydroxybutyrate, insulin, cortisol, GH, IGF-I, IGF-I binding protein (IGFBP)-1, IGFBP-3, leptin, and T_3. LH pulsatility was unaffected by an energy availability of 30 kcal/kg LBM·d ($P > 0.3$), but below this threshold LH pulse frequency decreased, whereas LH pulse amplitude increased (all $P < 0.04$). This disruption was more extreme in women with short luteal phases ($P < 0.01$). These incremental effects most closely resembled the effects of energy availability on plasma glucose, β-hydroxybutyrate, GH, and cortisol and contrasted with the dependencies displayed by the other metabolic hormones (simultaneously $P < 0.05$). These results demonstrate that LH pulsatility is disrupted only below a threshold of energy availability deep into negative energy balance and suggest priorities for future investigations into the mechanism that mediates the nonlinear dependence of LH pulsatility on energy availability. (*J Clin Endocrinol Metab* 88: 297–311, 2003)

THE PREVALENCE OF amenorrhea is elevated in women who restrict their diets and who are intensely physically active (1). The associated hypoestrogenism causes skeletal demineralization that increases the risk of stress fractures in the near term and of osteoporosis later in life (1). The United States military may face large liabilities for the medical care of servicewomen who develop such conditions as a result of their military service. Therefore, the United States military is interested to learn how to refine nutritional guidelines for physically active women so that servicewomen can better protect their reproductive and skeletal health while maintaining their fitness for physically demanding military missions.

In 1980, Warren (2) was the first to suggest that menstrual disorders in dancers are disrupted by an energy drain, but an empirically testable energy availability hypothesis was first clearly stated in terms of brain energy availability by Winterer *et al.* (3) in 1984. That hypothesis holds that failure to provide sufficient metabolic fuels to meet the energy requirements of the brain causes an alteration in brain function that disrupts the GnRH pulse generator through a mechanism yet to be identified.

Reproductive function in mammals does depend on the cellular availability of oxidizable metabolic fuels. In mammals, reproductive function is disrupted by dietary restriction, by administration of pharmacological inhibitors of oxidative metabolism, by administration of insulin that diverts blood glucose into storage and away from oxidative pathways, by cold exposure that consumes metabolic fuels in shivering and nonshivering thermogenesis, and by physical activity in which metabolic fuels are consumed in organized muscular contractions (4, 5).

Reproductive function critically depends on the pulsatile release of GnRH from GnRH neurons in the arcuate nucleus of the hypothalamus and, thereby, on the consequent, and more readily assessed, pulsatile release of LH by gonadotrophs in the pituitary. Previously, we experimentally disrupted LH pulsatility in regularly menstruating women by restricting their energy availability (operationally defined and behaviorally controlled as dietary energy intake minus exercise energy expenditure) to 10 kcal/kg lean body mass (LBM) per day (6, 7). As so defined, the term energy availability refers to the behavior of the subjects and not to the cellular availability of metabolic fuels inside them. Of course, by controlling energy availability, we expected to affect the cellular availability of metabolic fuels, but these were not under our direct control. In the same experiment, we also prevented the apparent suppressive effects of exercise stress on LH pulsatility by dietary supplementation, demonstrating that exercise has no suppressive effect on LH pulsatility beyond the impact of its energy cost on energy availability (6, 7). Similar effects on LH pulsatility have been reported in habitually active women when their exercise training regimen was increased while their dietary energy intake was reduced (8). Exercise-induced amenorrhea in female monkeys has also been reversed by dietary supplementation (9).

Our earlier experiments did not establish whether LH

Abbreviations: BBT, Basal body temperature; CEE, controlled exercise energy expenditure; E_2, estradiol; 24EB, 24-h energy balance; 24EE, 24-h energy expenditure; β-HOB; β-hydroxybutyrate; IRMA, immunoradiometric; LBM, lean body mass; PO_2, pressure of O_2; RER, respiratory exchange ratio; VO_{2max}, maximum oxygen uptake.

pulse frequency and amplitude are linearly proportional to energy availability, or whether LH pulsatility is disrupted abruptly at a particular threshold of energy availability. Nor did those experiments establish how much energy availability women require to prevent the disruption of LH pulsatility. Therefore, the primary objective of this experiment was to answer these questions.

Over the years, many metabolic substrates and hormones have been investigated as hypothetical signals of energy status to the GnRH pulse generator, but conflicting evidence has been presented for all of them (5, 10). Such hypotheses usually derived from observations of responses to a single extreme level of energy deficiency, often fasting, or pharmacological blockade or stimulation. Incremental dose-response relationships between energy availability and LH pulsatility and between energy availability and these metabolic hormones and substrates had not been quantified within the physiological range concurrently in the same subjects. Therefore, on the principle that causation implies association, the secondary objective of this experiment was to compare the incremental dose-response effects of energy availability on LH pulsatility to those on the metabolic substrates and hormones to seek guidance for future investigations into the mechanism mediating the effects on LH pulsatility.

In this randomized, repeated-measures, prospective cohort experiment, regularly menstruating, habitually sedentary, young women performed repeated trials in which diet and exercise were controlled for 5 d in the early follicular phase of the menstrual cycle. This report compares and contrasts the incremental effects of balanced (45 kcal/kg LBM·d) and restricted (10, 20, and 30 kcal/kg LBM·d) energy availability treatments on LH pulsatility and several metabolic hormones and substrates.

Subjects and Methods

Subject selection

Healthy, young, regularly menstruating women were recruited through posters and newspaper advertisements. All volunteers signed consent forms and received a full verbal and written description of the nature of the experiment, its associated risks and benefits, and their ability to withdraw from the experiment at any time. The protocol for this experiment was approved by the Institutional Review Boards of Ohio University, The Ohio State University, and the Surgeon General of the Department of the Army.

Volunteers were screened through interviews and questionnaires pertaining to their medical, dietary, and exercise histories, and through a physical examination performed by a consulting physician. Volunteers with histories or evidence of heart, liver, or kidney disease, diabetes, menstrual or thyroid disorders, pregnancy, lactation, and congenital or acquired orthopedic abnormalities were excluded. All subjects were required to be between 18 and 30 yr of age, nonsmokers, with no recent history of dietary restriction or athletic training. Hematocrits above 35% were required. Volunteers habitually performing more than 60 min/wk of aerobic exercise were excluded. On the basis of 7-d diet records, volunteers habitually consuming less than 35 or more than 55 kcal/kg LBM·d of energy were excluded. Body fatness determined by underwater weighing was required to be 18–30%.

Volunteers were also required to provide records of menstrual cycles occurring at regular intervals of 26–32 d for the previous 3 months and an average luteal length of at least 11 d as determined by an immunoradiometric (IRMA) assay of LH in daily urine samples. Women with short, long, and irregular menstrual cycles and with such short luteal phases were excluded to ensure that results were not skewed by preexisting reproductive disorders.

The demographic characteristics of the subjects are shown in Table 1. There were no differences in age, age of menarche, gynecological age, menstrual cycle length, luteal length, any parameter of body size or composition, maximal aerobic capacity, or habitual dietary energy intake between the women assigned to the three restricted energy availability treatments.

Experiment

Design. In this repeated-measures design (Fig. 1), subjects performed the experiment portion of the protocol (Fig. 2) twice, once at a balanced and once at one of three different restricted energy availability (A) treatments in random order to distinguish treatment effects from random individual variation and from fixed effects of participation in the experiment. All subjects performed X = 15 kcal/kg LBM (63 kJ/kg LBM) of 70% of maximum oxygen uptake (VO_{2max}) exercise each day for 5 d while their daily dietary energy intake (I) was controlled. In one group, dietary intake was controlled at I = 60 and 45 kcal/kg LBM·d (251 and 188 kJ/kg LBM·d) in separate treatments to provide balanced and restricted energy availabilities of A = I − X = 45 and 30 kcal/kg LBM·d (188 and 125 kJ/kg LBM·d), respectively. In a second group, dietary energy intake was controlled at I = 60 and 35 kcal/kg LBM·d (251 and 146 kJ/kg LBM·d) to provide balanced and restricted energy availabilities of A = I − X = 45 and 20 kcal/kg LBM·d (188 and 84 kJ/kg LBM·d). In the third group, dietary energy intake was controlled at I = 60 and 25 kcal/kg LBM (251 and 105 kJ/kg LBM·d) to provide balanced and restricted energy availabilities of A = I − X = 45 and 10 kcal/kg LBM·d (188 and 42 kJ/kg

TABLE 1. Demographic characteristics of the women who have received the balanced (45 kcal/kg LBM·d) and one of the three restricted energy availability treatments

Characteristics	Units	Restricted energy availability treatments (kcal/kg LBM · d)			*P*
		10	20	30	
n		10	11	8	
Calendar age	yr	21 ± 1	20 ± 1	22 ± 1	0.47
Age of menarche	yr	13 ± 0.4	13 ± 0.5	13 ± 0.4	0.78
Gynecological age	yr	8 ± 1	8 ± 1	9 ± 1	0.61
Menstrual cycle length	d	29.5 ± 0.6	30.2 ± 0.5	29.1 ± 0.4	0.34
Luteal length	d	12.6 ± 0.4	12.3 ± 0.5	12.8 ± 0.2	0.67
Height	cm	164.5 ± 2.0	162.6 ± 1.4	165.1 ± 2.3	0.61
Weight	kg	59.7 ± 1.3	59.1 ± 1.2	60.0 ± 1.9	0.92
Body fat	%	24.9 ± 1.2	26.2 ± 0.9	25.1 ± 1.4	0.68
LBM	kg	44.8 ± 1.2	43.6 ± 0.7	44.9 ± 1.6	0.60
VO_{2max}	ml O_2/kg BW · min	42.3 ± 1.4	38.3 ± 1.3	39.4 ± 1.3	0.10
Dietary intake	kcal/d	2000 ± 100	2000 ± 70	1830 ± 120	0.36
Dietary intake	kcal/kg LBM · d	45.8 ± 1.8	45.5 ± 1.5	40.6 ± 1.6	0.08

Data are presented as mean ± SE.

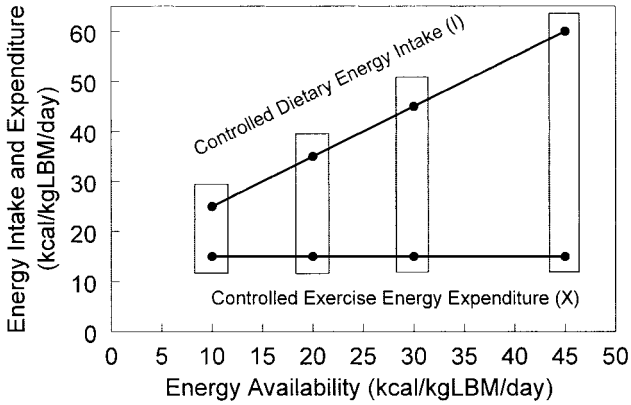

FIG. 1. Experimental design. Women were assigned to contrasting energy availability treatments of 45 and 10, 45 and 20, and 45 and 30 kcal/kg LBM·d. All subjects performed a CEE of 15 kcal/kg LBM·d in aerobic exercise at 70%VO_{2max} under supervision while their dietary energy intake was controlled to achieve the intended energy availability treatments.

LBM·d). Treatments were repeated at intervals of at least 2 months to allow time for full recovery from blood sampling.

Protocol. The 9-d experiment portion of the protocol began on the second to fifth day of the menstrual cycle, according to whether a subject's follicular phase in the screening cycle was shorter or longer. Experiment starting days did not differ by more than one cycle day between the two trials. Treatments were applied, and data and samples were collected 7 d/wk, as necessary. Each subject provided a urine sample and a blood sample between 0730 and 0830 h on the 3 pretreatment days and the 5 treatment days. Immediately after the treatments had been completed on the fifth treatment day, subjects were driven to the General Clinical Research Center (GCRC) at The Ohio State University Hospital where blood samples were drawn via a venous catheter at 10-min intervals for 24 h for the determination of LH pulsatility and the responses of various metabolic substrates and hormones. Subjects were not permitted to nap during the day during treatments or in the GCRC. In the GCRC, lights were turned off at 2230 h and turned on at 0630 h, and sleep onset and offset were observed and recorded.

Energy expenditure. During the 3 pretreatment days and the treatment days, each subject wore an accelerometric physical activity monitor (Tritrac, Hemokinetics, Madison, WI) during all waking hours, except while bathing, to estimate 24-h energy expenditure. Each morning, a laboratory staff member downloaded into a computer database the minute by minute record of data stored in the instrument during the previous 24 h. The total energy expenditure for each 24-h period (24EE) was calculated on each treatment day.

Controlled energy expenditure (CEE) was defined as the total energy expenditure during exercise as measured by indirect calorimetry. It was comprised of: 1) the subject's habitual waking energy expenditure, estimated by accelerometry, that would have occurred during the exercise time period if the subject had gone about her usual waking activities, plus 2) the CEE due to exercise (X = 15 kcal/kg LBM·d = 63 kJ/kg LBM·d). Habitual waking energy expenditure during the exercise time period was estimated as the average of the physical activity monitor records from 0800 to 2400 h on the first 2 pretreatment days.

Twenty-four-hour energy balance (24EB) was calculated as the difference between the controlled 24-h dietary energy intake (I) and total 24EE.

Control of treatments

Diet. A commercially available clinical dietary product (Ensure Plus, Ross Laboratories, Columbus, OH) was used to set energy intake for the selected levels of energy availability. The formula of this product is composed of 28% fats, 15% protein, and 57% carbohydrates. Subjects were instructed to eat all of the food they were provided and nothing else, except water, during the treatment period. Subjects were also provided with a daily multivitamin and mineral tablet. A daily qualitative urinary dipstick assay for the ketone acetoacetate was used as an indicator of noncompliance. Meals were administered at standardized times each day, including one meal consisting of one fifth to two fifths of the daily energy intake administered half an hour before each exercise session. During the 24-h frequent blood sampling sessions in the GCRC, three meals each, consisting of one third of the daily energy intake, were administered at standardized times after the restricted energy availability treatments at 10 kcal/kg LBM·d and 20 kcal/kg LBM·d. Four meals were administered at standardized times after the restricted treatment at 30 kcal/kg LBM·d and the balanced treatment at 45 kcal/kg LBM·d.

Energy intake (I), expenditure (X), and availability (A) were normalized by LBM to control the energy available to actively metabolizing tissue, regardless of individual differences in body composition.

Exercise. For the exercise treatment, subjects walked up a grade on a motorized treadmill ergometer under continuous supervision in 30- to 40-min sessions separated by 10-min rest periods. Exercise intensity was closely controlled by setting treadmill speed and grade, as required, to elicit 70% of each individual's maximal oxygen consumption measured during the selection portion of the protocol. During the first exercise session, submaximal oxygen uptake was calculated from measurements of expired respiratory gases, and the associated heart rate and respiratory exchange ratio (RER) were recorded. During later exercise sessions, oxygen consumption was measured while making minor adjustments to treadmill speed and grade, as necessary, to maintain this oxygen consumption. Because the energy cost of exercise per liter of oxygen consumed depends on substrate use, the total duration of each individual's daily exercise was adjusted according to the individual's rate of exercise energy expenditure as indicated by the individual's oxygen uptake and RER. For this purpose, oxygen uptake and RER were measured for 5–8 min during each exercise session.

Effectiveness of treatment administration. The balanced and three restricted energy availability treatments actually administered to the subjects are described in Table 2. By design, there were no differences between the exercise regimens (%VO_{2max}, CEE, and X) administered to the subjects in any of the energy availability treatments. By contrast and also by design, the controlled dietary energy intakes (I) and, thereby, the energy availabilities (A) and 24EBs of the subjects during the three restricted energy availability treatments were extremely different from those in the balanced energy availability treatment (all $P < 10^{-7}$), and from one another, whereas 24EB was indistinguishable from zero ($P = 0.80$) during the balanced energy availability treatment.

Experimental data collection

These data were obtained by RIA, IRMA, and enzymatic assay of blood samples collected during the experiment. The three baseline (daily pretreatment) blood samples were assayed for E_2. Frequent blood samples collected in the GCRC were assayed for LH; those collected at half-hour intervals were assayed for glucose; and a pooled sample collected at 30-min intervals was assayed for FSH, T_3, insulin, IGF-I, IGF-I binding protein (IGFBP)-1, and IGFBP-3. A pooled sample collected at 10-min intervals was assayed for GH, E_2, leptin, and cortisol. In addition, two pooled samples collected at 10-min intervals during the feeding part of the day (0950 to 2250 h) and during the fasting part of the day (2300 to 0940 h) were assayed for GH, insulin, and cortisol. The 0800 h blood sample collected in the GCRC was assayed for serum β-hydroxybutyrate (βHOB).

Assays. All assays were performed in duplicate. LH, FSH, and GH were assayed with an IRMA Allegro procedure produced by Nichols Institute Diagnostics (San Juan Capistrano, CA). The intra-assay and interassay coefficients of variation for these assays in our laboratory were: LH, 4% and 9% at 3.8 IU/liter; FSH, 3% and 9% at 4.4 IU/liter; and GH, 3% and 6% at 1.6 μg/liter, respectively. LH standards were calibrated against the World Health Organization First International Reference Preparation (1st IRP 68/40). IGFBP-1 and IGFBP-3 were assayed with IRMA kits, and E_2 was assayed with an ultrasensitive RIA kit from Diagnostic Systems Laboratories, Inc. (Webster, TX). Cortisol and T_3 were assayed with RIA kits produced by Diagnostics Products (Los Angeles, CA). Insulin was assayed with a supersensitive RIA kit from Linco Research, Inc. (St.

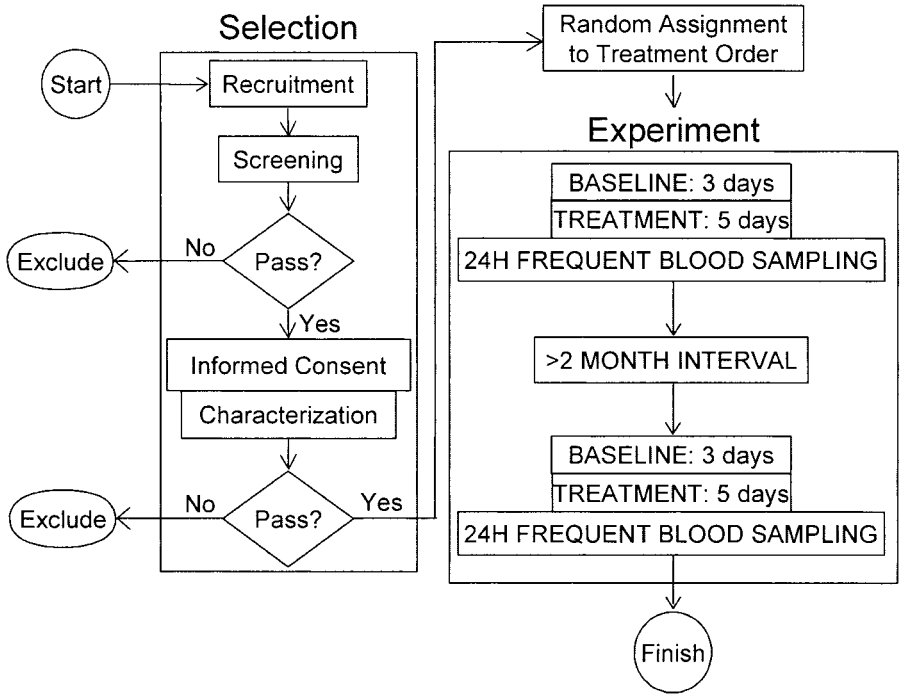

FIG. 2. Experimental protocol. Volunteers were required to pass an extensive selection process before they were admitted as subjects to the experiment. In the experiment, subjects completed two energy availability treatments that were preceded by baseline metabolic substrate and hormone measurements and followed by 24-h determinations of metabolic substrate and hormone profiles and LH pulsatility. The two energy availability treatments were administered in a random order and separated by at least 2 months for recovery from blood loss.

Charles, MO). Leptin was assayed with an RIA kit from Linco Research, Inc. IGF-I was assayed with an RIA kit from Nichols Institute Diagnostics using an acid-ethanol precipitation extraction step. The intra-assay and interassay coefficients of variation for assays of E_2, cortisol, insulin, leptin, and IGF-I were: E_2, 4% and 4% at 210 pmol/liter; cortisol, 3% and 10% at 290 nmol/liter; T_3, 4% and 5% at 1.1 nmol/liter; insulin, 5% and 12% at 55 pmol/liter; leptin, 5% and 8% at 8 ng/ml; and IGF-I, 3% and 12% at 270 ng/ml, respectively. The intra-assay and interassay coefficients of variation for the IGFBP assays were: IGFBP-1, 2% and 10% at 53 ng/ml; and IGFBP-3, 2% and 8% at 3500 ng/ml, respectively.

Plasma glucose was assayed enzymatically with a YSI Model 23L Glucose/Lactate Analyzer (YSI, Inc., Yellow Springs, OH). Intra-assay and interassay coefficients of variation for this assay were 1% and 3% at 4.7 mmol/liter, respectively. Serum β-HOB was assayed enzymatically by a kit from Sigma (St. Louis, MO). Intra-assay and interassay coefficients of variation for this assay were 3% and 7% at 900 μmol/liter, respectively.

Data analysis

Carbohydrate availability. We defined carbohydrate availability observationally as an outcome variable as the difference between dietary carbohydrate intake and the amount of carbohydrate oxidized during exercise beyond the requirements of normal activity. For each individual, the amount of carbohydrate oxidized during exercise was calculated from the RER and energy expenditure measured during each exercise session. Carbohydrate oxidation during normal activity was estimated using the RER measured during a separate resting session and the subject's habitual energy expenditure during her usual waking activities during the exercise time period, as estimated by the physical activity monitor.

LH pulsatility. The time series of LH concentrations over 24 h were analyzed for 24-h mean concentration, pulse frequency, and pulse amplitude by the computer program Cluster (11). The author (A.B.L.) has used this copyrighted program extensively in previous studies of LH pulsatility (6, 7, 12) and has the programmer's written permission to possess and use it for research. A 2×1 pulse configuration was used with up and down T-ratios of 2.5 to give a 2.1% false-positive pulse detection rate.

Substrate and hormone analysis. For each subject, the difference between serum β-HOB concentrations measured at 0800 h in the GCRC after the restricted and balanced energy availability treatments was calculated as the effect of energy availability.

Twenty-four-hour means of GH, FSH, T_3, insulin, cortisol, leptin, IGF-I, IGFBP-1, IGFBP-3, and E_2 were determined from pooled samples, as were means from the samples pooled during the feeding phase (0950–2250 h) and the fasting phase (2300–0940 h) of the day. Twenty-four-hour transverse means of LH and plasma glucose were calculated from the 24-h time series determined from the frequent blood samples, as were transverse means for plasma glucose from the feeding and fasting phases of the day.

Statistical analysis. All data sets were tested for non-normality, heteroscedasticity, and outliers before statistical hypothesis tests were performed. Outliers were rejected, and data sets were transformed as necessary. One-sided, single-sample tests were used to detect effects of low energy availability at 30, 20, and 10 kcal/kg LBM·d; and repeated-measures ANOVA with *post hoc* single-sided, two-sample least significant difference tests were performed to compare the effects of these restricted energy availability treatments. Single-sided tests were employed, because the direction of interest in outcome variables was known in advance.

We were interested in effects of energy availability in the directions of effects and differences that had been identified previously. For example, we had reported LH pulse frequency to be reduced and LH pulse amplitude to be increased in regularly menstruating athletes compared with regularly menstruating sedentary women (12), and we had induced such effects in sedentary women by restricting their dietary energy intake (6) and in exercising women by increasing their exercise energy expenditure (7). Meanwhile, metabolic hormone and substrate abnormalities in athletes are consistently indicative of chronic energy deficiency. Therefore, we used paired, single-sided t tests to detect and to quantify the incremental effects of restricted energy availability on LH pulsatility and on metabolic hormones and substrates.

For exploratory purposes, to prioritize particular metabolic parameters for future investigation of their potential role in the mechanism of the disruption of LH pulsatility by low energy availability, we compared the shapes of the incremental responses of the metabolic substrates and hormones to those of LH pulse frequency and amplitude, both visually and statistically. By visual inspection, we compared the linearity, nonlinearity, and the number of inflections in these curves. Statistically, we analyzed the incremental responses as follows. First, to overcome differences in the units, signs, and magnitudes of the responses, we nor-

TABLE 2. The paired balanced and three restricted energy availability treatments

Parameters	Restricted energy availability treatments (kcal/kg LBM · d)			Balanced 45 kcal/kg LBM · d	ANOVA *P* (between treatments)
	10	20	30		
Daily exercise					
% VO_{2max}	69.6 ± 0.6	70.1 ± 0.2	70.6 ± 0.2	70.4 ± 0.2	0.33
% HR_{max}	89.8 ± 1.2**	84.5 ± 1.5**	83.8 ± 1.0	83.7 ± 0.9	0.25
RPE	14.8 ± 0.6	14.8 ± 0.4	14.9 ± 0.6	14.3 ± 0.3	0.93
Duration (min/d)	102 ± 3.0	114 ± 3.7	111 ± 4	108 ± 2.4	0.58
Controlled exercise energy expenditure (CEE)					
kcal/d	825 ± 20	830 ± 20	850 ± 30	840 ± 13	0.80
kcal/kg LBM · d	18.4 ± 0.2	19.2 ± 0.2	19.0 ± 0.2	18.8 ± 0.1	0.86
Exercise energy expenditure (EEE)					
kcal/d	690 ± 30	660 ± 10	690 ± 30	670 ± 10	0.35
kcal/kg LBM · d	15.3 ± 0.4	15.3 ± 0.0	15.4 ± 0.1	15.1 ± 0.1	0.29
24-h Energy expenditure (24EE)					
kcal/d	2660 ± 50	2610 ± 40	2640 ± 100	2670 ± 40	0.73
kcal/kg LBM · d	59.6 ± 1.1	60.0 ± 0.8	58.9 ± 1.2	60.3 ± 0.7	0.78
Controlled dietary energy intake (CDI)					
kcal/d	1120 ± 30***	1520 ± 20***	2020 ± 70***	2670 ± 40	$<10^{-7}$
kcal/kg LBM · d	25.0 ± 0.01***	34.9 ± 0.1***	45.0 ± 0.1***	60.2 ± 0.2	$<10^{-7}$
Energy availability (CDI − EEE)					
kcal/d	430 ± 20***	850 ± 10***	1330 ± 50***	2000 ± 30	$<10^{-7}$
kcal/kg LBM · d	9.6 ± 0.4***	19.6 ± 0.1***	29.7 ± 0.1***	45.1 ± 0.2	$<10^{-7}$
24-h Energy balance: (CDI − 24EE)					
kcal/d	−1540 ± 30***	−1090 ± 30***	−620 ± 50***	−1 ± 30	$<10^{-7}$
kcal/kg LBM · d	−34.6 ± 1.1***	−25.1 ± 0.9***	−13.9 ± 1.2***	−0.1 ± 0.7	$<10^{-7}$
24-h Carbohydrate availability					
kcal/d	160 ± 15***	360 ± 15***	530 ± 30***	910 ± 20	$<10^{-7}$
g/d	40 ± 4***	90 ± 4***	130 ± 7***	230 ± 5	$<10^{-7}$
kcal/kg LBM · d	3.5 ± 0.3***	8.1 ± 0.3***	11.8 ± 0.5***	20.3 ± 0.3	$<10^{-7}$

Exercise energy expenditure (EEE) was calculated as the controlled total energy expenditure during exercise (CEE = 18 kcal/kg LBM · d) minus the portion of the 24-h energy expenditure (24EE) occurring during the exercise time period on three pretreatment days. Values are presented as mean ± SE (1 kcal = 4.182 kJ). HR, Heart rate; RPE, rating of perceived exertion.

Difference between restricted and balanced (45 kcal/kg LBM · d) energy availability treatments: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

malized the incremental effects of restricted energy availability on all of these parameters as percentages of their responses at 10 kcal/kg LBM·d. This normalization also eliminated variance at 10 kcal/kg LBM·d. Because variance at 45 kcal/kg LBM·d had already been eliminated by taking each subject as her own control, the ensuing ANOVA was reduced to the intermediate incremental effects at 20 and 30 kcal/kg LBM·d in common units on a standard percentage scale. In this analysis, we analyzed the sum of the differences at 20 and 30 kcal/kg LBM·d between the percentage responses of LH pulse frequency and each of the 10 metabolic substrates and hormones, and also for differences between the percentage responses of LH pulse amplitude and the 10 metabolic parameters, as well as for the differences between the percentage responses of LH pulse frequency and amplitude.

Statistical power. Paired data sets were obtained at a balanced energy availability of 45 kcal/kg LBM·d and at restricted energy availability treatments of 10, 20, and 30 kcal/kg LBM·d from 10, 11, and 8 women, respectively. These numbers of observations provided sufficient statistical power to detect changes of 1.0, 1.0, and 1.2 SD values, respectively, in single-sided, single-sample tests at $100\alpha = 5\%$ and $100\beta = 10\%$ probabilities of type I and type II errors, and differences of 1.3, 1.4, and 1.4 SD values in single-sided, two-sample comparisons between 10 and 20, 20 and 30, and 10 and 30 kcal/kg LBM·d, respectively, at the same error rates.

In the ANOVA comparing the shapes of the incremental effects of restricted energy availability on LH pulsatility and on metabolic substrates and hormones, the number of subjects and the one-way classification of 21 comparisons provided a $100\alpha = 5\%$ probability of a type I error in concluding that there were differences between the shapes of the responses. In the subsequent Student's *t* tests with a Bonferroni correction for 21 planned comparisons, the number of observations provided $100\alpha = 5\%$ probability of a type I error in concluding simultaneously that the shapes of particular responses differed from one another (13).

Results

Effects of restricted energy availability on body weight

The restricted energy availability treatments at 10, 20, and 30 kcal/kg LBM·d reduced body weight by 2.0 ± 0.3 kg (3.4% of body weight), 1.1 ± 0.2 kg (1.8% of body weight), and 1.3 ± 0.3 kg (2.1% of body weight), respectively. The balanced energy availability treatment had no effect on body weight ($P = 0.2$).

Effects of restricted energy availability on carbohydrate availability

The effects of the balanced and restricted energy availability treatments on carbohydrate availability are shown in Table 2. These treatments and the body's responses to them in altering the use of metabolic fuels by muscle during exercise led to extreme differences in carbohydrate availability between the energy availability treatments (all $P < 10^{-7}$). The restricted energy availability treatments at 10, 20, and 30 kcal/kg LBM·d reduced carbohydrate availability by approximately 80%, 60%, and 40%, respectively.

Effects of restricted energy availability on 24-h LH pulsatility

The main finding of this experiment is that the restricted energy availability treatments at 10 and 20 kcal/kg LBM·d suppressed LH pulse frequency and increased LH pulse amplitude (all $P < 0.04$), but the restricted energy availability

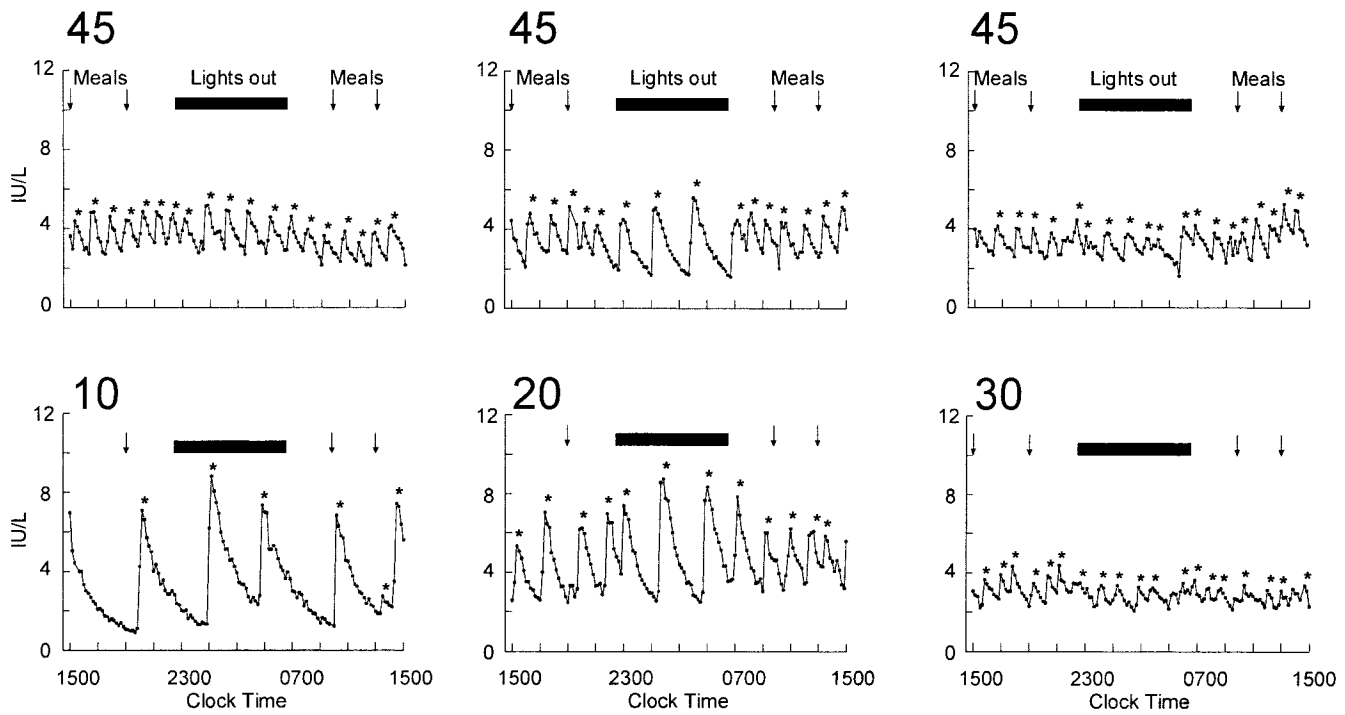

FIG. 3. LH pulse profiles. Twenty-four-hour LH pulse profiles for representative women after the balanced energy availability treatments of 45 kcal/kg LBM·d (*top*) and the paired restricted energy availability treatments of 10, 20, and 30 kcal/kg LBM·d, *left* to *right*, respectively (*bottom*). *Asterisks* indicate LH pulses. The *black bar* indicates when lights were turned off. *Arrows* indicate meals.

TABLE 3. Restricted energy availability treatment effects on LH pulsatility

Parameter	Units	Restricted energy availability treatment effects (kcal/kg LBM · d)			Balanced 45 kcal/kg LBM · d	ANOVA *P* (between treatment effects)
		10	20	30		
24-h LH pulse frequency	p/24h	$-5.8 \pm 1.6^{**P^{(a)=0.06}}$	$-3.0 \pm 1.0^{**P^{(b)=0.06}}$	0.0 ± 1.0^{cc}	17.7 ± 0.6	0.01
24-h LH pulse amplitude	IU/liter	$1.7 \pm 0.5^{**aa}$	$0.5 \pm 0.2^{*}$	0.1 ± 0.2^{cc}	1.5 ± 0.1	0.01
24-h LH mean	IU/liter	-0.4 ± 0.3	-0.4 ± 0.4	0.2 ± 0.4	3.8 ± 0.3	0.47

Treatment effects are paired differences within individuals: restricted − balanced. Values are expressed as mean ± SE. Statistical tests were single-sided by design.

Significance of restricted treatment effects: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Significance of differences between restricted treatment effects: 10 *vs.* 20 kcal/kg LBM · d: $^{a}P < 0.05$, $^{aa}P < 0.01$, $^{aaa}P < 0.001$; 20 *vs.* 30 kcal/kg LBM · d: $^{b}P < 0.05$, $^{bb}P < 0.01$, $^{bbb}P < 0.001$; 10 *vs.* 30 kcal/kg LBM · d: $^{c}P < 0.05$, $^{cc}P < 0.01$, $^{ccc}P < 0.001$.

treatment at 30 kcal/kg LBM·d had no effect on either LH pulse frequency or amplitude (both $P > 0.3$).

Figure 3 shows representative LH pulse profiles during the final 24 h of the balanced energy availability treatment at 45 kcal/kg LBM·d and of the three restricted energy availability treatments at 10, 20, and 30 kcal/kg LBM·d. There were no differences between LH pulse frequency, LH pulse amplitude, and 24-h LH mean concentration over 24 h during the balanced energy availability treatment displayed by the women in the three restricted energy availability treatment groups. Therefore, their LH pulsatility data in the balanced energy availability treatment are pooled for reference in Table 3. LH pulsatility data were statistically analyzed pairwise within subjects, however, to determine the incremental effects of the restricted energy availability treatments, which are also listed in Table 3. No level of restricted energy availability had any effect on the 24-h LH mean concentration.

The pattern of these effects, illustrated in Fig. 4A, shows the threshold dependence of LH pulsatility on energy availability. In comparison to the reference LH pulse frequency and amplitude measured after the balanced energy availability treatment of 45 kcal/kg LBM·d, the restriction of energy availability had no effect on 24-h LH pulse frequency and amplitude until energy availability fell below 30 kcal/kg LBM·d. Restricting energy availability to 20 kcal/kg LBM·d suppressed 24-h LH pulse frequency by 16% ($P < 0.01$) and increased 24-h LH pulse amplitude by 21% ($P < 0.05$). Restricting energy availability to 10 kcal/kg LBM·d further suppressed 24-h LH pulse frequency by 39% and increased 24-h LH pulse amplitude by 109% (both $P < 0.01$).

After the above results had been determined, we noticed that the distributions of LH pulsatility effects at restricted energy availabilities of 10 and 20 kcal/kg LBM·d were strongly bimodal, with almost entirely nonoverlapping subgroups having larger and smaller effects. This observation led to a search for other parameters measured in the exper-

iment that might be similarly distributed. Because our primary interest in this search was to identify inexpensive and convenient predictors of increased susceptibility to reproductive disruption by low energy availability, the first variables we checked were the screening and descriptive characteristics listed in Table 1 (calendar age, age of menarche, gynecological age, menstrual cycle length, luteal length, height, weight, body fatness, LBM, aerobic power, dietary intake) as well as variance in menstrual cycle length. The next variables we checked were the metabolic substrate and hormone parameters measured after the balanced energy availability treatment listed in Table 4, because these might resemble values measured in outpatients by primary care physicians. Only then did we check the metabolic substrate and hormone responses to restricted energy availability listed in Table 4, because the detection of these effects requires an expensive and highly controlled experimental protocol.

Of all these parameters, only one was bimodal like the effects of restricted energy availability on LH pulsatility, and the significance of the association was extreme ($P < 10^{-7}$): *i.e.* luteal phase length. Figure 4B illustrates how the effects of restricted energy availability on LH pulse frequency ($P = 0.01$ and $P = 0.01$) and amplitude ($P < 0.01$ and $P = 0.31$) at 10 and 20 kcal/kg LBM·d, respectively, were significantly more extreme in subjects with 11-d luteal phases than in subjects with longer luteal phases. Thirty-one percent of the subjects in the experiment had 11-d luteal phases.

Effects of restricted energy availability were marginally and inconsistently significant in the subgroups with longer luteal phase length ($P = 0.08$ at 10 kcal/kg LBM·d and $P = 0.03$ at 20 kcal/kg LBM·d for LH pulse frequency; and $P = 0.07$ at 10 kcal/kg LBM·d and $P = 0.13$ at 20 kcal/kg LBM·d for LH pulse amplitude).

Effects of restricted energy availability on sleep-wake differences in LH pulsatility

LH pulse frequency was 20% slower during sleep after the balanced energy availability treatment ($P = 10^{-5}$) and after the restricted energy availability treatment at 30 kcal/kg LBM·d ($P < 0.01$), but it was 30% slower during sleep after the 20 kcal/kg LBM·d treatment ($P < 0.001$). Initially, a slowing of LH pulse frequency during sleep was not detected after the 10 kcal/kg LBM·d treatment, but when we discarded the data from the two subjects with the most extreme reductions in LH pulse frequency after this treatment (to only 3 and 6 pulses per 24 h, which made the detection of sleep-wake differences in them very unreliable), a 40% slower LH pulse frequency during sleep was detected in the remaining subjects after this treatment, also ($P = 0.02$). With respect to the independent question of the effect of energy availability on LH pulse frequency during the waking and sleeping hours separately, the restricted energy availability treatments at 10 and 20 kcal/kg LBM·d slowed LH pulse frequency during both the sleeping and waking hours, but 30 kcal/kg LBM·d did not slow LH pulse frequency during either sleeping or waking hours.

Effects of restricted energy availability on FSH and E_2

No level of restricted energy availability had any effect on the 24-h mean FSH concentration (mean = 4.9 IU/liter; all

TABLE 4. Restricted energy availability treatment effects on metabolic hormones and substrates

Hormones and substrates	Units	Restricted energy availability treatment effects (kcal/kg LBM/d)			Balanced 45 kcal/kg LBM · d	ANOVA *P* (between treatment effects)
		10	20	30		
Glucose						
24 h	mmol/liter	$-0.7 \pm 0.1^{***aaa}$	$-0.3 \pm 0.1^{***}$	$-0.1 \pm 0.1^{**ccc}$	5.0 ± 0.04	$<10^{-4}$
1000–2230 h	mmol/liter	$-0.7 \pm 0.2^{***aaa}$	0.03 ± 0.1	0.0 ± 0.1^{ccc}	5.4 ± 0.06	$<10^{-3}$
2300–0930 h	mmol/liter	$-0.8 \pm 0.1^{***a}$	$-0.6 \pm 0.1^{***bb}$	$-0.3 \pm 0.1^{**ccc}$	4.6 ± 0.06	10^{-4}
β-HOB	μmol/liter	$1890 \pm 280^{***aaa}$	$1020 \pm 110^{***bb}$	$230 \pm 60^{**ccc}$	120 ± 10	$<10^{-4}$
Insulin						
24 h	pmol/liter	$-73 \pm 7^{***a}$	$-44 \pm 9^{***}$	$-32 \pm 11^{**cc}$	99 ± 6	0.01
0950–2250 h	pmol/liter	$-120 \pm 14^{***aa}$	$-69 \pm 12^{***}$	$-43 \pm 13^{**ccc}$	150 ± 10	0.002
2300–0940 h	pmol/liter	$-18 \pm 3^{***a}$	$-9 \pm 2^{**}$	-4 ± 4^{cc}	29 ± 2	0.01
Cortisol						
24 h	nmol/liter	$50 \pm 10^{***aaa}$	$20 \pm 10^{*}$	$10 \pm 5^{**ccc}$	170 ± 10	$<10^{-3}$
1000–2230 h	nmol/liter	$40 \pm 10^{**aa}$	0.0 ± 10	10 ± 10^{c}	140 ± 10	0.05
2300–0930 h	nmol/liter	$50 \pm 10^{***}$	$30 \pm 10^{**}$	$20 \pm 5^{**cc}$	180 ± 10	0.04
GH						
24 h	μg/liter	$0.9 \pm 0.3^{**}$	$0.6 \pm 0.1^{**b}$	0.0 ± 0.2^{cc}	2.6 ± 0.2	0.04
0950–2250 h	μg/liter	$0.9 \pm 0.3^{**}$	$0.8 \pm 0.2^{**}$	0.4 ± 0.3	2.2 ± 0.2	0.35
2300–0940 h	μg/liter	$1.1 \pm 0.3^{**a}$	0.4 ± 0.3^{b}	-0.7 ± 0.4^{ccc}	3.0 ± 0.2	0.005
IGF-I	ng/ml	$-99 \pm 14^{***}$	$-80 \pm 14^{***bb}$	-20 ± 15^{ccc}	260 ± 20	0.003
IGFBP-1	ng/ml	$49 \pm 4^{***aaa}$	$28 \pm 4^{***}$	$20 \pm 5^{**ccc}$	28 ± 2	$<10^{-3}$
IGF-I/IGFBP-1		$-9 \pm 2^{***}$	$-8 \pm 2^{**}$	$-6 \pm 2^{**}$	12 ± 2	0.68
IGFBP-3	ng/ml	$-310 \pm 120^{*}$	$-290 \pm 70^{***bb}$	240 ± 180^{cc}	2930 ± 120	0.008
IGF-I/IGFBP-3		$-0.03 \pm 0.003^{***}$	$-0.02 \pm 0.004^{***b}$	$-0.01 \pm 0.003^{**ccc}$	0.09 ± 0.005	0.008
T_3	nmol/liter	$-0.2 \pm 0.03^{***}$	$-0.2 \pm 0.04^{**bb}$	$-0.07 \pm 0.03^{*c}$	1.1 ± 0.04	0.01
Leptin	ng/ml	$-6.1 \pm 0.8^{***}$	$-4.8 \pm 0.8^{***}$	$-3.0 \pm 0.7^{**cc}$	8.8 ± 0.7	0.05

Treatment effects are paired differences within individuals: restricted − balanced. Values are expressed as mean ± SE.

Significance of restricted treatment effects: * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Significance of differences between restricted treatment effects: 10 *vs.* 20 kcal/kg LBM · d, ^{a} $P < 0.05$, ^{aa} $P < 0.01$, ^{aaa} $P < 0.001$; 20 *vs.* 30 kcal/kg LBM · d, ^{b} $P < 0.05$, ^{bb} $P < 0.01$, ^{bbb} $P < 0.001$; 10 *vs.* 30 kcal/kg LBM · d, ^{c} $P < 0.05$, ^{cc} $P < 0.01$, ^{ccc} $P < 0.001$.

$P > 0.4$). On the baseline days, E_2 levels measured at 0800 h were similar both between the groups ($P = 0.47$) and within the individuals ($P = 0.74$, $P = 0.28$, and $P = 0.92$) assigned to the pairs of balanced and restricted energy availability treatments at 10, 20, and 30 kcal/kg LBM·d, respectively (grand mean = 71 pmol/liter). The separate pooled 24-h mean E_2 concentrations measured in the GCRC were suppressed 15% (from mean = 95 to 79 pmol/liter, $P < 0.01$) by the restricted 10 kcal/kg LBM·d energy availability treatment, but not by the restricted 20 kcal/kg LBM·d (mean = 104 and 101 pmol/liter; $P = 0.39$) and 30 kcal/kg LBM·d (mean = 98 and 103 pmol/liter; $P = 0.4$) treatments.

Effects of restricted energy availability on metabolic substrates and hormones

The women assigned to the three restricted energy availability treatment groups displayed only one difference in metabolic substrate and hormone concentrations during the balanced energy availability treatment: the women administered the 20 kcal/kg LBM·d restricted energy availability treatment displayed higher 24-h serum cortisol levels during the balanced energy availability treatment (200 ± 10 *vs.* 170 ± 10 and 150 ± 10 nmol/liter, both $P < 0.001$). Because all of these cortisol values were within the normal physiological range, however, all of the subjects' metabolic substrate and hormone data during the balanced energy availability treatment are pooled for reference in Table 4. The results of the paired, within-subject, incremental effects of the restricted energy availability treatments on metabolic substrates and hormones are also listed in Table 4.

The 10, 20, and 30 kcal/kg LBM·d restricted energy availability treatments reduced 24-h mean plasma glucose concentrations by 15% ($P < 0.001$), 5% ($P < 0.001$), and 3% ($P < 0.01$), respectively. The similar ($P = 0.14$) effects at 20 and 30 kcal/kg LBM·d were 57% ($P = 0.01$) and 86% ($P = 0.0001$) smaller than the effect at 10 kcal/kg LBM·d. Reductions in plasma glucose at 10 kcal/kg LBM·d ($P < 0.001$) occurred during both the feeding (1000–2230 h) and fasting (2300–0930 h) phases of the day (both $P = 0.001$), whereas the reductions at 20 and 30 kcal/kg LBM·d occurred only during the fasting phase ($P < 0.001$ and $P < 0.01$, respectively).

The 10, 20, and 30 kcal/kg LBM·d restricted energy availability treatments increased fasting morning β-HOB by approximately 1600% ($P < 0.0001$), 1000% ($P = 0.0001$), and 300% ($P = 0.01$), respectively. The effect at 20 kcal/kg LBM·d and the smaller ($P = 0.01$) effect at 30 kcal/kg LBM·d were 47% ($P < 0.01$) and 87% ($P < 0.0001$) smaller than that at 10 kcal/kg LBM·d.

The incremental effects of restricted energy availability on 24-h mean plasma glucose and fasting morning β-HOB are illustrated in Fig. 4A. By visual inspection of linearity, nonlinearity, and number of inflections, the shapes of these curves resemble those of the incremental effects of restricted energy availability on LH pulse frequency and amplitude.

Twenty-four-hour mean insulin concentrations were suppressed linearly by restricted energy availability (all $P < 0.05$), with the suppression occurring primarily during the feeding phase of the day (0950–2250 h). The effects at 20 and 30 kcal/kg LBM·d were 40% ($P < 0.05$) and 56% ($P < 0.001$) smaller than the effect at 10 kcal/kg LBM·d.

The restricted energy availability treatments elevated 24-h mean serum cortisol levels in a nonlinear pattern. Small similar ($P = 0.20$) increases in 24-h mean serum cortisol at 20 kcal/kg LBM·d and 30 kcal/kg LBM·d increased another 150% ($P < 0.001$) with the further reduction in energy availability to 10 kcal/kg LBM·d. Thus, most of the cortisol response to restricted energy availability occurred below 20 kcal/kg LBM·d, and, like glucose and in contrast to insulin, it occurred primarily during the fasting part of the day

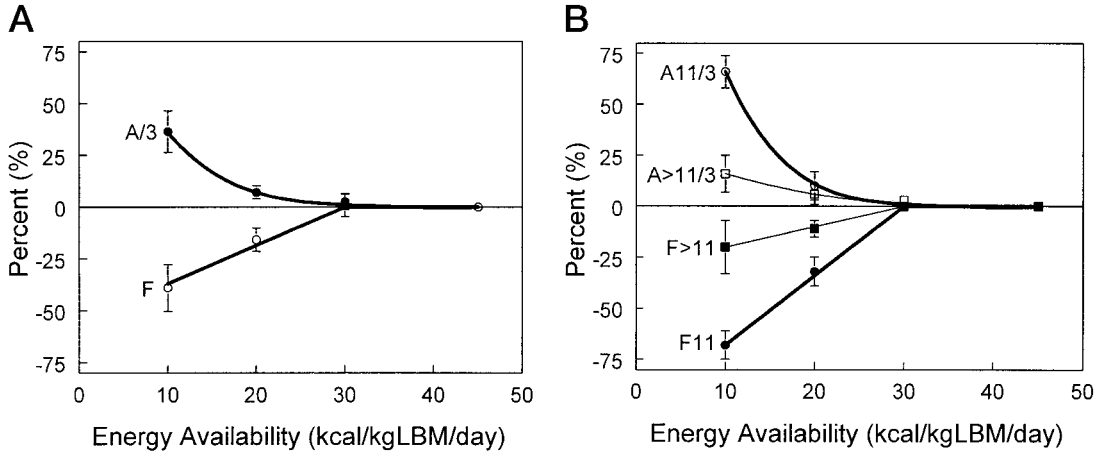

FIG. 4. A, Incremental effects of energy availability on LH pulse amplitude (●, *top*) and LH pulse frequency (○, *bottom*). Effects are expressed relative to values at 45 kcal/kg LBM·d. Effects on LH pulse amplitude have been divided by three for graphical symmetry. As energy availability declines from energy balance at approximately 45 kcal/kg LBM·d, effects begin at a threshold at approximately 30 kcal/kg LBM·d and become more extreme as energy availability is further reduced below 20 kcal/kg LBM·d. B, Incremental effects of energy availability on LH pulse amplitude (□, ○, *top*) and LH pulse frequency (■, ●, *bottom*) in subgroups of women with luteal phases of 11 d (○, ●) and more than 11 d (□, ■) as determined by urinary LH surge determination. Women with shorter luteal phases (<11 d) had been excluded from participation in the experiment. Effects are relative to values at 45 kcal/kg LBM·d. Effects on LH pulse amplitude have been divided by three for graphical symmetry. Women with luteal phases of 11 d were substantially more susceptible to disruption of LH pulsatility by restricted energy availability than were women with longer luteal phases. A, Amplitude; F, frequency.

(2300–0930 h); at 20 and 30 kcal/kg LBM·d, it occurred only during the fasting phase of the day.

The incremental effects of restricted energy availability on 24-h mean plasma insulin and 24-h mean cortisol are illustrated in Fig. 5B. By visual inspection, the shape of incremental effects of restricted energy availability on 24-h mean cortisol resembles those on 24-h mean plasma glucose, fasting morning β-HOB, LH pulse frequency, and amplitude. All of these parameters have little or no response at 30 kcal/kg LBM·d and increasing responses at lower energy availabilities. By contrast, insulin levels declined linearly with energy availability.

Like LH pulse amplitude and frequency, GH and IGF-I were disrupted at a threshold of energy availability not higher than 30 kcal/kg LBM·d ($P < 0.01$ and $P < 0.001$, respectively). Unlike LH pulsatility, however, the incremental effects of restricted energy availability on GH and IGF-I did not become more extreme because energy availability was further reduced to 10 kcal/kg LBM·d ($P = 0.14$ and $P = 0.20$, respectively). Restricted energy availability had opposite effects on IGFBP-1 and IGFBP-3, with IGFBP-1 increasing 175% whereas IGFBP-3 decreased 11% at an energy availability of 10 kcal/kg LBM·d. Again, these effects occurred at a threshold of energy availability not higher than 30 kcal/kg LBM·d (both $P < 0.001$). Consequently, the ratios of IGF-I to IGFBP-1 and IGFBP-3, which are indices of free IGF-I, displayed different incremental responses to restricted energy availability. Although both ratios were suppressed at 30 kcal/kg LBM·d (both $P < 0.01$), IGF-I/IGFBP3 was suppressed linearly, like insulin, whereas all three levels of restricted energy availability suppressed IGF-I/IGFBP-1 similarly ($P = 0.68$).

The incremental effects of restricted energy availability on GH, IGF-I, IGF-I/IGFBP-1, and IGF-I/IGFBP-3 are illustrated in Fig. 5C. The percentage changes in Fig. 5C and the absolute changes in Table 4 tell somewhat different stories about bioactive IGF-I (*i.e.* the percentage reductions in IGF-I/IGFBP-1 do differ at the three levels of restricted energy availability). By visual inspection, the shapes of these curves are different from the shapes of incremental effects of restricted energy availability on LH pulse frequency and amplitude. The response of IGFI/IGFBP-3 is linear like insulin, the curvature of IGFI/IGFBP-1 is opposite to that of LH pulse frequency and amplitude, and the curves for IGF-I and GH have two inflections at 20 and 30 kcal/kg LBM·d, although the inflection in the curve for GH at 20 kcal/kg LBM·d is not as strong as that in the curve for IGF-I. So, of these four somatotrophic parameters, the shape of the GH response is most similar to those of LH pulse frequency and amplitude.

Unlike LH pulse amplitude and frequency, T_3 was substantially suppressed by a restricted energy availability of 30 kcal/kg LBM·d ($P < 0.05$). T_3 was further suppressed by a reduction in energy availability to 20 kcal/kg LBM·d ($P < 0.01$), but there was no further incremental effect when energy availability was further reduced to 10 kcal/kg LBM·d ($P = 0.55$), also unlike the incremental effects of restricted energy availability on LH pulsatility.

Leptin, too, was suppressed by a restricted energy availability of 30 kcal/kg LBM·d ($P < 0.01$). The incremental effects of further reductions in energy availability to 20 and 10 kcal/kg LBM·d on the absolute concentrations of leptin displayed in Table 4 were ambiguous ($P = 0.07$ and $P = 0.11$, respectively), in contrast to the obvious and progressively more extreme effects of restricted energy availability on LH pulsatility. The incremental effects of these levels of restricted energy availability on the percentage changes in leptin displayed in Fig. 5D were obviously significant, however ($P < 0.001$ and $P < 0.05$, respectively).

Thus, by visual inspection, the shapes of the incremental effects of restricted energy availability on T_3 and leptin shown in Fig. 5D were also different from the shapes of those on LH pulse frequency and amplitude shown in Fig. 4 and from the patterns of incremental effects on the metabolic substrates shown in Fig. 5A. The response of T_3 has two inflections like IGF-I, and the response of leptin has the opposite curvature, like IGF-I/IGFBP-1.

The results of the statistical analysis comparing the shapes of the incremental effects of restricted energy availability on LH pulsatility and on the metabolic substrates and hormones is illustrated in Fig. 6. By normalization and repeated-measures control, all of the normalized responses were exactly 0% and 100% at 45 and 10 kcal/kg LBM·d, respectively. Figure 6A shows the normalized responses of LH pulse frequency and amplitude. At 20 and 30 kcal/kg LBM·d, these responses were not significantly different from one another. Figure 6B shows the normalized responses of 24-h mean glucose, cortisol, and GH in comparison to that of LH pulse frequency. At 20 and 30 kcal/kg LBM·d, these responses were not significantly different from one another. Figure 6C shows the normalized responses of glucose in comparison to that of LH pulse amplitude. At 20 and 30 kcal/kg LBM·d, these responses were not significantly different from one another. Figure 6D shows the normalized responses of the other metabolic hormone parameters in comparison to those of LH pulse frequency and amplitude. At 20 and 30 kcal/kg LBM·d, these normalized metabolic parameters were all significantly different from those of LH pulse frequency and amplitude (simultaneously $P < 0.05$).

Discussion

By administering balanced and three levels of restricted energy availability under tightly controlled conditions in this experiment, we quantified the dependence of LH pulsatility and of selected metabolic hormones and substrates on energy availability in healthy, young, regularly menstruating, habitually sedentary women. For the purpose of this experiment, energy availability was defined operationally and controlled behaviorally as dietary energy intake minus exercise energy expenditure. We emphasize, therefore, that, as defined by us, the term energy availability refers to the behavior of the subjects and not to the cellular availability of metabolic fuels inside them. Of course, by quantifying and controlling energy availability, we expected to affect the cellular availability of metabolic fuels, but these were not under our direct control.

This experiment yielded three main findings: 1) LH pulsatility was disrupted abruptly at a threshold of energy availability not higher than 30 kcal/kg LBM·d; 2) the disruptive effects of subthreshold energy availability were bimodal, with substantially larger effects occurring in the subjects with

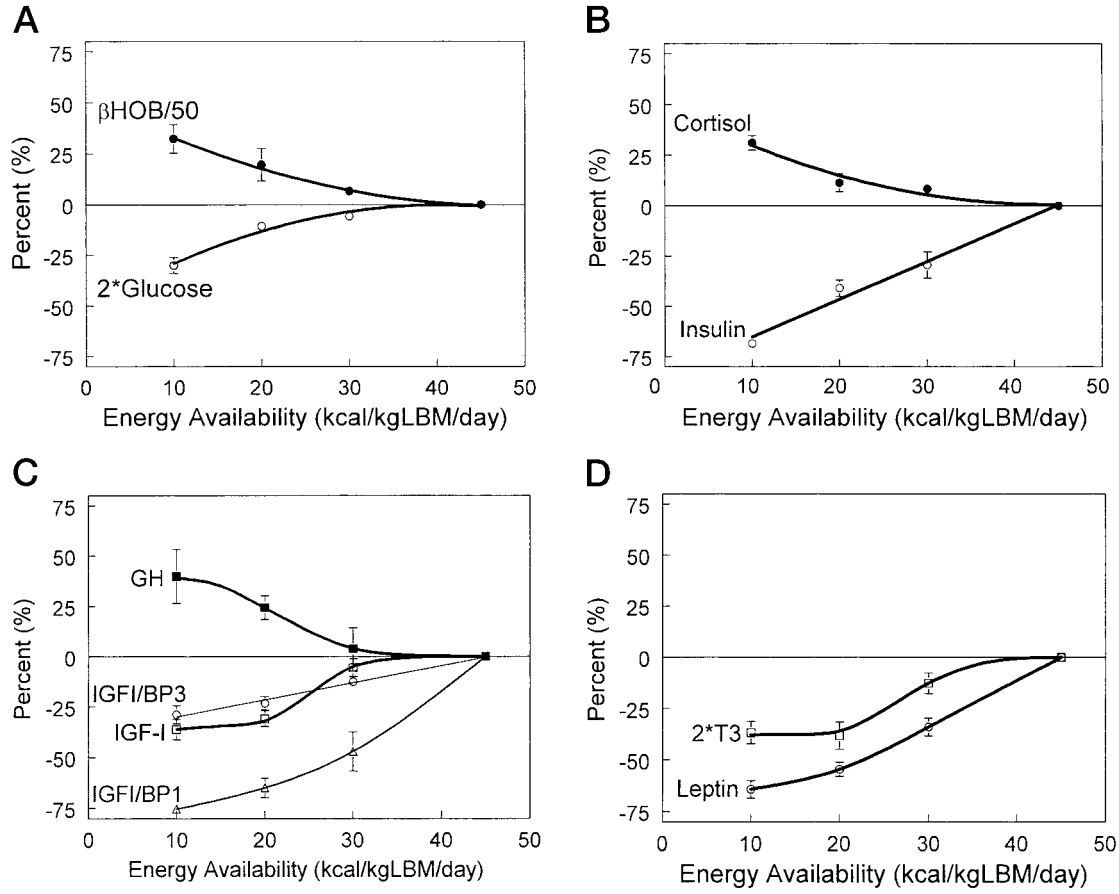

FIG. 5. Incremental effects of restricted energy availability on metabolic substrates and hormones. A, Incremental effects on the metabolic substrates β-HOB (●, *top*) and plasma glucose (○, *bottom*). Effects are shown relative to values at 45 kcal/kg LBM·d. Effects on β-HOB have been divided by 50, and effects on plasma glucose have been doubled for graphical symmetry. Effects on β-HOB and glucose become progressively more extreme as energy availability decreases. B, Incremental effects on the metabolic hormones cortisol (●, *top*) and insulin (○, *bottom*). Effects are shown relative to values at 45 kcal/kg LBM·d. Insulin declines linearly with energy availability, whereas effects on cortisol become progressively more extreme as energy availability decreases. C, Incremental effects on the somatotrophic metabolic hormones GH (■, *top*) and IGF-I (□, *bottom*) and the ratios IGF-I/IGFBP-1 (△) and IGF-I/IGFBP-3 (○). Effects are shown relative to values at 45 kcal/kg LBM·d. Effects on GH and IGF-I tend to flatten out below 20 kcal/kg LBM·d as GH resistance becomes more extreme. Both estimates of bioactive IGF-I have declined significantly and substantially at 30 kcal/kg LBM·d. D, Incremental effects on the metabolic hormones T_3 (□, *top*) and leptin (○, *bottom*). Effects are shown relative to values at 45 kcal/kg LBM·d. The effect on T_3 is doubled for graphical clarity. Both T_3 and leptin have declined significantly and substantially at 30 kcal/kg LBM·d. These effects tend to flatten out below 20 kcal/kg LBM·d.

the shortest luteal phases (11 d); and 3) the incremental effects of restricted energy availability on LH pulsatility most closely resembled those on glucose, β-HOB, GH, and cortisol.

Subject selection and experimental controls

Because the subjects assigned to the three restricted energy availability treatments in this experiment (Table 1) were indistinguishable in age, menstrual history, body size and composition, aerobic fitness, or dietary habits, subject selection and assignment are unlikely to have biased experimental results. Moreover, these subjects are physically representative of the general population, because their body size and composition were very similar to those of the reference woman of Behnke and Wilmore (14), a theoretical model based upon the average physical dimensions obtained from detailed measurements of thousands of individuals who were subjects in large-scale anthropometric surveys.

The narrow range of menstrual cycle length among the subjects admitted to the experiment reflects the investigators' intention to select subjects with a high degree of menstrual regularity to ensure that results were not skewed or obscured by preexisting reproductive disorders. Volunteers with short luteal phases [10 d or less as determined by the timing of the LH surge, corresponding to 9 d or less as determined by basal body temperature (BBT)] were excluded from the experiment, because such short luteal phases are associated with increased risks of infertility. However, an unintended and unexpected characteristic of the women admitted to the experiment was the large proportion with 11-d luteal phases as determined by the timing of the LH surge (31%). By contrast, only 9% of biphasic cycles of the same range in length (26–32 d) and only 15% of biphasic cycles in women of the same range in gynecological age (5–15 yr) display the corresponding 10-d luteal phases as determined by BBT (15). This serendipitous characteristic of our subject sample led to the second major finding of this experiment.

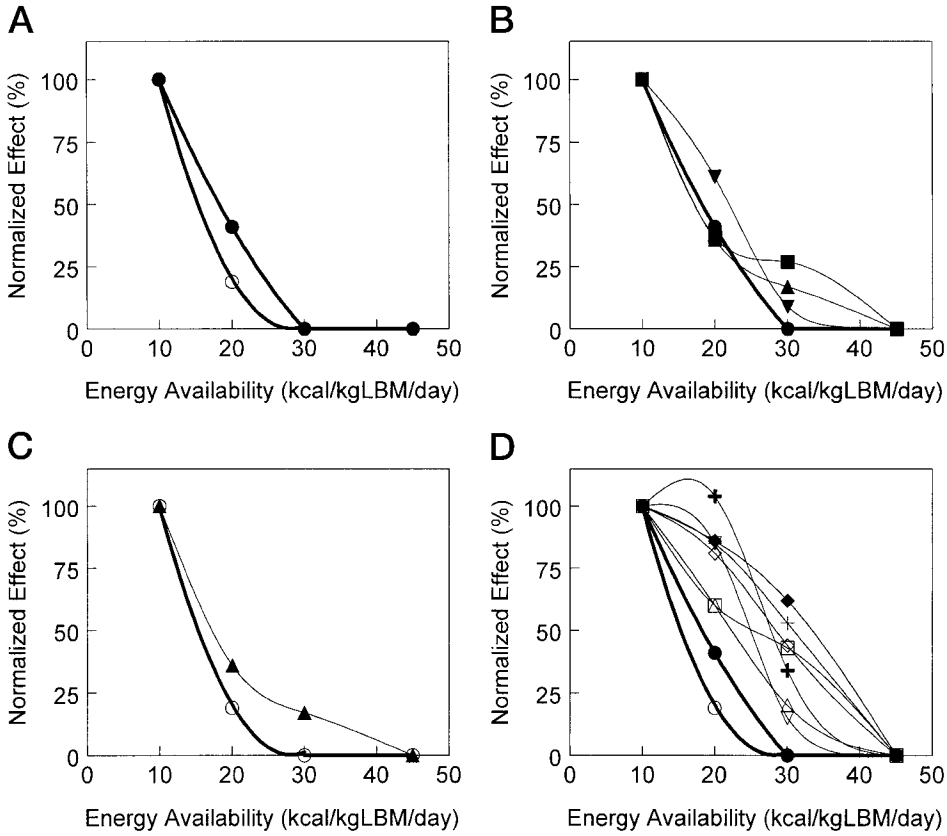

FIG. 6. Normalized incremental effects of restricted energy availability on LH pulsatility and on metabolic substrates and hormones. By normalization and repeated-measures control, all of the normalized responses were exactly 0% and 100% at 45 and 10 kcal/kg LBM·d, respectively. A, Comparison of the normalized incremental effects on LH pulse frequency (●) and amplitude (○). Intermediate incremental effects at 20 and 30 kcal/kg LBM·d were not significantly different from one another. B, Comparison of the normalized incremental effects on 24-h plasma glucose (▲), cortisol (■), and GH (▼) to those on LH pulse frequency (●). Intermediate incremental effects at 20 and 30 kcal/kg LBM·d were not significantly different from one another. C, Comparison of normalized incremental effects on 24-h plasma glucose (▲) to those on LH pulse amplitude (○). Intermediate incremental effects at 20 and 30 kcal/kg LBM·d were not significantly different from one another. D, Comparison of normalized incremental effects on β-HOB (△), insulin (□), IGF-I (▽), IGF-I/IGFBP-1 (♦), IGF-I/IGFBP-3 (◇), T_3 (✚), leptin (+) to those on LH pulse frequency (●) and amplitude (○). Intermediate incremental effects at 20 and 30 kcal/kg LBM·d for these metabolic parameters were significantly different from those of LH pulse frequency and amplitude (simultaneously $P < 0.05$). Of these metabolic parameters, the least significantly different one was β-HOB (△).

The controlled dietary and exercise treatments (Table 2) were administered precisely according to the experimental design (Fig. 1). The difference in exercise energy expenditure between balanced and restricted energy availability treatments was much less than 1%. The difference between balanced energy availability treatments administered to the three restricted energy availability treatment groups was less than 2%; and the restricted energy availabilities administered were within 4% of the planned treatments. Therefore, variance in the measured outcomes is comprised almost entirely of variance in subject responses, with negligible confounding by variance in the treatments themselves. Consequently, the experimental results can be reliably attributed to the planned contrasts between the balanced (45 kcal/kg LBM·d) and restricted (10, 20, and 30 kcal/kg LBM·d) energy availability treatments.

Threshold effect of energy availability on LH pulsatility

The maintenance of normal LH pulsatility, despite a 33% reduction in energy availability at 30 kcal/kg LBM·d in this experiment, demonstrates for the first time that LH pulsatility is not linearly proportional to energy availability. Rather, the disruption of LH pulsatility occurs at a threshold of energy availability not higher than 30 kcal/kg LBM·d (~1330 kcal/d for our 45 kg LBM subjects).

By the term threshold we do not mean to imply an "all-or-nothing" response like that of the neuron action potential, but rather a "something-or-nothing" response like the lactate threshold in exercise physiology, the hypoxic threshold in ventilatory physiology, and the glycemic thresholds in blood glucose regulation. As exercise intensity increases, for example, nothing happens to blood lactate concentration until oxygen uptake exceeds a certain level that depends upon the size and physical fitness of the person. Beyond that level, blood lactate concentration rises linearly. Similarly, as arterial pressure of O_2 (PO_2) decreases, nothing happens to pulmonary ventilation, which continues to be regulated by pressure of CO_2, until arterial PO_2 reaches approximately 60 mm Hg, below which point pulmonary ventilation begins to rise with further decreases in arterial PO_2. Similarly, too, as en-

ergy availability declines from energy balance at approximately 45 kcal/kg LBM·d, nothing happens to LH pulsatility until energy availability reaches 30 kcal/kg LBM·d (*i.e.* to −15 kcal/kg LBM·d with respect to energy balance), below which point effects on LH pulsatility begin to be observed.

In Fig. 4, we drew the simplest (*i.e.* least abrupt) model of two straight lines to represent this something-or-nothing threshold relationship with effects beginning at 30 kcal/kg LBM·d. Additional data at 15 and 25 kcal/kg LBM·d might confirm this something-or-nothing threshold model, or they might motivate a more complicated (*i.e.* more abrupt) all-or-nothing threshold model closer to 20 kcal/kg LBM·d.

Of course, more prolonged experiments are needed to quantify the incremental effects of chronically low energy availability on LH pulsatility and ovarian function in women. In anticipation of those results, however, it is noteworthy that 6 yr of 30% dietary restriction had no effect on reproductive hormones or menstrual cyclicity in monkeys (16). Recent cross-sectional comparisons of estimated energy availability in athletes are also consistent with this interpretation: amenorrheic athletes were estimated to habitually self-administer an energy availability of 16 kcal/kg LBM·d, whereas cyclic athletes habitually self-administered 30 kcal/kg LBM·d (17). Thus, although the precise location and steepness of the energy availability threshold between 20 and 30 kcal/kg LBM·d remains to be determined, 30 kcal/kg LBM·d appears to be sufficient energy availability to prevent the disruption of LH pulsatility and ovarian function.

This quantity has practical applicability, because 30 kcal/kg LBM·d was the energy availability of the subjects performing the exercise energy expenditure administered in this experiment (15 kcal/kg LBM·d) with the dietary energy intake of energy-balanced sedentary women (45 kcal/kg LBM·d). Because total controlled exercise energy expenditure during the exercise treatment in this experiment was approximately 840 kcal, this suggests that many women may be able to maintain normal LH pulsatility while running up to approximately 8 miles/d —if they do not simultaneously reduce their dietary energy intake compared with energy-balanced sedentary women. If they do reduce their dietary energy intake, as many exercising women do, then they risk falling below the energy availability threshold. We emphasize, however, that our findings in this short-term study of LH pulsatility will need to be confirmed by similarly controlled, longer-term studies of ovarian function and by intervention studies with athletes before firm conclusions can be drawn about the effectiveness of particular dietary interventions for preventing and treating athletic amenorrhea.

One wonders how such a threshold effect of energy availability on LH pulsatility would provide an evolutionary advantage for humans. It has long been speculated that the suppression of fertility in times of energy deficiency provides such an evolutionary advantage by focusing available food resources on the existing population. How would not suppressing reproductive function in times of small energy deficiency provide a further evolutionary advantage? Perhaps this preservation of fertility in times of slight energy deficiency would be necessary if these deficiencies occurred often, indeed every night in the fast that occurs during prolonged hours of sleep.

Effects of energy availability on sleep-wake differences in LH pulse frequency

The slowing of LH pulse frequency during sleep in presumably 24-h energy-balanced women in the follicular phase has been well established. We found that the degree of slowing during sleep was unaffected by a reduction of energy availability from 45 to 30 kcal/kg LBM·d, but that it increased from −20% to −30% at 20 kcal/kg LBM·d and appeared to increase further to −40% at 10 kcal/kg LBM·d when the obscuring influence of the most extremely disrupted subjects at 10 kcal/kg LBM·d had been removed. Restricted energy availability slowed LH pulsatility during both the waking and sleeping hours at 10 and 20 kcal/kg LBM·d, but during neither the waking nor the sleeping hours at 30 kcal/kg LBM·d. Thus, the reduction of energy availability to 30 kcal/kg LBM·d not only had no effect on 24-h LH pulsatility but also had no effect on LH pulsatility during the separate waking and sleeping hours, and consequently on the degree to which LH pulsatility slows during sleep.

Association of energy availability effects with luteal phase length

The larger disruption of LH pulsatility by restricted energy availability in women with 11-d luteal phases in this experiment suggests that such women might be at higher risk than others for the suppression of ovarian function and skeletal demineralization by energy deficiency. If this were confirmed through further research, luteal length would be a convenient and inexpensive means for the military and athletic teams to screen female recruits to identify those needing special attention to ensure that their dietary and exercise regimens provide sufficient energy availability to preserve normal reproductive function.

With only six subjects in each of the subgroups with longer luteal phases, the power of the statistical tests that did not find effects on LH pulsatility to be consistently significant at subthreshold energy availabilities was limited. If more subjects are studied in these subgroups in the future, consistent effects might be detected in them, but these effects would be small.

Many years ago, we observed that amenorrheic runners and triathletes had similar athletic training regimens to those of athletes with highly regular menstrual cycles that were symptomatically indistinguishable from those of sedentary women (12). Upon endocrine investigation, however, the highly regular menstrual cycles in these athletes displayed short luteal phases with substantially reduced progesterone levels. Others reported a 3-month sample incidence of such luteal phase deficiency in cyclic athletes to be as high as 79% (18). We have speculated that luteal suppression in athletes might be explained three ways (19). First, luteal suppression might be an intermediate condition that progresses to amenorrhea under more prolonged or severe circumstances. We were and we remain skeptical of this hypothesis, because the athletic histories of our cyclic and amenorrheic athletes were so similar in intensity and duration.

Second, luteal suppression might be the endpoint of a more successful acclimation to athletic training. Certainly, abruptly imposed, intensive athletic training has quickly in-

duced menstrual disorders, whereas gradually imposed, moderate training has not (20, 21), but because energy availability was not precisely measured and controlled in those experiments, we do not yet know whether they confounded the rate of change in athletic training with differences in energy availability.

Third, we speculated that luteal suppression may be the endpoint of rigorous training regimens in women with more robust reproductive systems. This experiment presents the first evidence that the reproductive systems in some women may, indeed, be more robust against energy deficiency than those in other women. Luteal phases shorter than 10 d as determined by BBT (<11 d as determined by the LH surge) occur in 35% of biphasic cycles of women in the general population with gynecological ages of 5–15 yr (15). This proportion of women at potentially increased risk of reproductive disruption by energy restriction drops to only 18% among slightly older women with gynecological ages of 16–20 yr (15). Thus, the frequent observation that the prevalence of amenorrhea in athletes declines with age may reflect the age-related decline in the prevalence of short luteal phases. Further research will be needed to distinguish the independent effects of age and luteal length on the susceptibility of the reproductive system to disruption by energy deficiency.

Effects of energy availability on metabolic substrates and hormones

The incremental effects of restricted energy availability on LH pulse frequency and amplitude in this experiment were similar but opposite in sign. By visual inspection, these effects most closely resembled those on plasma glucose, and β-HOB and cortisol. By statistical analysis, the incremental effects on LH pulse frequency and amplitude most closely resembled those on plasma glucose, cortisol, and GH. These associations between demonstrated causal effects of restricted energy availability on LH pulsatility and on metabolic substrates and hormones do not prove that any of the metabolic substrates and hormones are involved in the mechanism mediating the effects of energy availability on LH pulsatility, but they do suggest priorities for future research into that mechanism. In particular, we do not expect metabolic parameters whose incremental responses to energy availability are most different from those of LH pulsatility (*i.e.* with large responses at 30 kcal/kg LBM·d where LH pulsatility is not disrupted and with smaller or no further responses at 10 and 20 kcal/kg LBM·d where LH pulsatility is disrupted) to play the most dominant roles in the mechanism mediating it.

Down to an energy availability of approximately 30 kcal/kg LBM·d, the responses of various metabolic hormones (insulin, cortisol, IGF-I/IGFBP-1, IGF-I/IGFBP-3, leptin, and T_3) maintained plasma glucose levels to within 3% of normal values. Below approximately 30 kcal/kg LBM·d; however, plasma glucose levels fell (by ~15% at 10 kcal/kg LBM·d), and β-HOB levels rose (by ~1600% at 10 kcal/kg LBM·d), despite larger responses of these hormones, and this is the range of energy availability at which effects on LH pulsatility appear. Below 20 kcal/kg LBM·d, the responses of most of the metabolic hormones (GH, IGF-I, IGF-I/IGFBP-1, IGF-I/IGFBP-3, T_3, and leptin) approached an asymptotic limit, whereas others (cortisol and insulin) clearly continued to increase. Despite the exaggerated responses of these counter-regulatory hormones, further deviations in the metabolic substrates and LH pulsatility occurred in tandem.

Unlike most other tissues, which exhibit considerable flexibility in the metabolic fuels that they use for energy, the brain is restricted almost exclusively to glucose as its substrate for energy metabolism (22). Under normal conditions, rates of brain glucose uptake are not rate limiting for brain metabolism, in that roughly twice as much glucose is transported across the blood-brain barrier as is needed to satisfy brain energy needs (23), the balance being transported back into the circulation. As arterial plasma glucose declines, however, only sufficient glucose arrives in the brain to satisfy the brain's energy demands, and below this level glucose transport across the blood-brain barrier becomes rate limiting for brain metabolism (24).

The glucose requirement of the adult human female brain is approximately 80 g/d (assuming a 60-kg female may be normalized by body mass to a 70-kg male requiring ~94 g/d; Ref. 25). This glucose requirement of the brain, which has virtually no glucose storage capacity of its own, exceeds both liver glycogen stores (~75 g glucose; Ref. 26) and the 24-h liver glycogenolytic capacity (~30 g glucose; Ref. 25). As a result, liver glycogen stores are almost entirely exhausted by fasting in less than 3 d (27), which is why we prolonged our experimental treatments for 5 d.

Because brain energy expenditure is constant over a wide range of brain activity (28), whereas muscle energy expenditure can increase by a factor of more than 200 during exercise (29), working muscle competes aggressively against the brain for plasma glucose. During a marathon, for example, working muscle consumes as much energy (~2350 kcal at ~90 kcal/mile) in 2–3 h as the brain consumes in 1 wk. During the controlled exercise in this experiment, working muscle oxidized more glucose in less than 2 h than the brain does in an entire day. Therefore, in addition to the glucose requirement of working muscle during exercise, daily dietary carbohydrate intake in this experiment would have had to provide the brain's entire daily glucose requirement plus the entire daily glucose requirement of normally active muscle (~30 g/d in a 70-kg male at rest; ~25 g/d in a 60-kg female). We found LH pulsatility was disrupted at a level of carbohydrate availability between 90 and 130 g/d of glucose, values bracketing the sum of the daily glucose requirements of the human brain (~80 g/d) and normally active muscle (~25 g/d).

Glucose-sensitive neurons containing neuropeptide Y in the arcuate nucleus of the hypothalamus, where the GnRH pulse generator is also localized (30–32), are activated by fasting (33), due in part to reduction in the inhibitory effects of insulin (34), leptin (35), and glucose (36). The firing rate of these neurons varies dramatically as local glucose concentrations are changed from 1–10 mmol/liter (32) and from 5–20 mmol/liter (37), but it has been much more difficult to show that variations in glucose levels within the physiological range influence neuroendocrine mechanisms that regulate physiological systems. Alterations in plasma glucose of

2 mmol/liter altered brain glucose levels by 0.2–0.3 mmol/liter, and this changed the firing rate of glucose-sensing neurons (38), but even 2 mmol/liter changes in plasma glucose are far greater than the 0.5 mmol/liter changes postulated to trigger meal initiation (39). In this experiment, restricted energy availability treatments that reduced 24-h mean plasma glucose levels by 0.1, 0.3, and 0.7 mmol/liter caused highly significantly different effects on LH pulse frequency and amplitude that were doubtless secondary to different effects on the GnRH pulse generator.

Under various circumstances (starvation, nursing, a ketogenic diet, diabetes, prolonged exercise, and other conditions that accelerate the mobilization and catabolism of fat), the human brain also metabolizes ketones for energy in proportion to their concentrations in the blood (40). It had been suggested that ketones might be a much more important fuel for brain energy metabolism and, thereby, for survival in mammals with high brain-to-body weight ratios (~2% in humans and ~1% in other primates) than in other mammals, who have much lower brain-to-body weight ratios (~0.1%; Ref. 41). This hypothesis was confirmed by observations that ATP production derived from brain ketone uptake during fasting was 10 times higher in humans (42) than in rats (43). After 3 d of fasting, ketone oxidation provided 35% of brain energy in humans. We are unaware of any reports of ketone-sensitive neurons in the brain, but receptors in the liver that are sensitive to the oxidation of fatty acids, glycerol, and ketones are thought to alter hepatic vagal afferent input to the central nervous system (for review, see Ref. 10).

Most glucose-sensitive neurons in the brain react in the same way to glucose and lactate (44). In healthy, fasting men and women, brain lactate is elevated, potentially due to ketones displacing brain lactate oxidation (45). Under insulin clamp conditions, thresholds for counter-regulatory hormone responses, hypoglycemic symptoms, and cognitive dysfunction are all reduced to lower glucose concentrations by infusions of either β-HOB or lactate (46). Plasma lactate levels rise as high as 30 mmol/liter during strenuous exercise (47), and brain lactate uptake has been shown to increase by a factor of 20 during exercise and to remain elevated during recovery (48). Therefore, lactate production during strenuous exercise may actually help to sustain rather than suppress LH pulsatility in women, over and above the beneficial effect of reduced muscle glucose utilization, in restricted energy availability conditions (7).

Previously, we had determined for the first time the independent effects of energy availability and exercise stress on serum cortisol and found that the stress of exercise has no effect on serum cortisol (or on T_3, GH, IGF-I, or insulin) beyond the impact of the energy cost of exercise on energy availability (7). For this reason and because all energy availability treatments in this experiment included the same exercise regimen, we expected the effects of energy availability on serum cortisol in this experiment to reflect the counter-regulatory function of cortisol. Indeed, the incremental effects of energy availability on serum cortisol mirrored the incremental effects of energy availability on plasma glucose.

In this experiment, cortisol was increased during both the feeding and fasting portions of the day by the energy availability treatment at 10 kcal/kg LBM·d, but only during the fasting part of the day by the energy availability treatments at 20 and 30 kcal/kg LBM·d. These findings were reminiscent of observations in amenorrheic athletes, in whom cortisol is elevated during both phases of the day, and in regularly menstruating, luteally suppressed athletes, in whom cortisol is elevated only at night (49).

Considerable animal research indicates that GnRH neurons are disturbed by activation of the hypothalamic-pituitary-adrenal axis via pathways involving CRH via endogenous opioid and pro-opiomelanocortin-derived peptides, or by increased cortisol negative feedback (50, 51). Such experiments have induced extreme activations of the adrenal axis, however, raising cortisol by several hundred percent, in contrast to the mild elevations seen in this experiment and in amenorrheic athletes (12, 49) and hypothalamic amenorrhea patients (52). The extent to which such physiological elevations in cortisol influence the GnRH pulse generator independently of the influence of plasma glucose is unknown.

The various hormones that participate in glucose counter-regulation by mobilizing metabolic fuels are activated at different something-or-nothing glycemic thresholds. As plasma glucose declines, first insulin begins to fall, then glucagon and epinephrine begin to rise, then GH begins to rise, and lastly cortisol begins to rise (53). Thus, the activation of particular counter-regulatory hormones reflects the degree of glucose deficiency, and the similarity of the incremental responses of GH and cortisol to those of LH pulse frequency and amplitude may imply only that plasma glucose levels have to fall below some glycemic threshold before they disrupt LH pulsatility.

In summary, the present experiment has contributed new qualitative and quantitative information about the dependence of LH pulsatility on energy availability in regularly menstruating young women. The disruption of LH pulsatility over 24 h, during the waking hours and during the sleeping hours, occurred at a threshold of energy availability not higher than 30 kcal/kg LBM·d. This disruption was greater in subjects with the shortest luteal phases, suggesting a simple method for identifying women especially susceptible to the disruption of reproductive function by energy deficiency. The incremental effects on LH pulse frequency and amplitude caused by restricted energy availability most closely resembled the incremental effects on plasma glucose, β-HOB, GH, and serum cortisol.

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Address all correspondence and requests for reprints to: Anne B. Loucks, Department of Biological Sciences, Ohio University, Athens, Ohio 45701. E-mail: loucks@ohiou.edu.

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