Modified activity-stress paradigm in an animal model of the female athlete triad

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The female athlete triad is a syndrome occurring in physically active women of reproductive age that is characterized by the interrelated effects of restrictive eating patterns, amenorrhea, and a greater risk for developing musculoskeletal injury, stress fracture, and long-term complications of osteoporosis (68). The concept of “energy drain” from increased exercise and restrictive eating has been suggested as an adaptive response and the underlying mechanism in athletic amenorrhea. According to the “energy drain” hypothesis, female athletes may be predisposed, or at greater risk, for developing menstrual disorders because of lower energy reserves that are also coupled with greater energy demands (55, 57, 86).

Studies on athletes have shown that exercise-related menstrual dysfunction is difficult to induce with exercise alone (22, 47, 58, 65, 70, 86, 91). Physical activity by itself, without other predisposing factors, has been increasingly questioned as a cause of reproductive dysfunction. The pathophysiology of exercise-related amenorrhea is complex and appears to involve interacting mechanisms that regulate body composition, energy balance, and stress-related variables associated with exercise intensity, restricted diet intake, and weight loss. Athletic activity seems to compound the effects of inadequate nutrition and low body weight (11, 30, 32, 86). High-volume training combined with caloric restriction may predispose women to exercise-induced changes in luteinizing hormone (LH) pulse frequency, while adequate caloric intake may prevent these changes (56, 58, 59, 92). In a series of eloquent studies, Loucks and colleagues examined the role of energy availability and intense exercise on LH pulsatility in nonathletic women. They conclude that the results of these studies contradict the hypothesis that LH pulsatility is disrupted by exercise alone and suggest that LH pulsatility is dependent on energy availability (56, 59).

Females with low body weight and associated decreases in adipose tissue, and those diagnosed with eating disorders and amenorrhea, have also been shown to reduce central and peripheral concentrations of leptin (6, 26, 38, 51). Kopp and coworkers (51) reported low leptin levels and amenorrhea in nonathletic women who were low in body weight. In these women, leptin concentrations were found to be a better predictor of amenorrhea than body mass index (BMI), fat mass, or percent body fat. Furthermore, these researchers claimed that a critical level of leptin might be needed to maintain normal menstrual function. More recently, low serum levels of leptin associated with restrained eating patterns and oligo-amenorrhea have been reported in female athletes (39, 52, 76).

Investigation of the female athlete triad poses a wide range of complications in sampling and research design because of the complexity of the disorder and the influence of multiple variables (68, 95). Homogeneous populations of female athletes with similar physical characteristics, training regimens, and lifestyle patterns are difficult to study. In addition, research guidelines for human participation place limitations on the experimental design and restrict manipulation of variables that may compromise the health and well-being of the participant. Animal research offers promise for studying the female athlete triad. In contrast to the human menstrual cycle, the 4- or 5-day estrous cycle of the rat is repeated approximately six to seven times per month (54, 61). Likewise, bone tissue turnover occurs at a faster rate in the rat (10–15 days) compared with humans (90 days) (33). Because of the increased turnover of biological systems, investigation of estrous cycling and bone mineral density (BMD) in rats can be advantageous for the researcher, providing an opportunity for repeated trials within an accelerated timeline. Although there is no animal model for

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the female athlete triad, characteristics of the syndrome have previously been developed in the laboratory rat (7, 10, 17, 19, 20, 27, 28, 33, 49, 62, 81, 83, 87, 97).

The classic activity-stress paradigm of restrictive feeding and wheel running activity in rats has been studied extensively over the past 40 years. This phenomenon has previously been referred to as “self-starvation,” “semistarvation-induced hyperactivity,” “activity-stress ulcer,” and “activity-based or anxiety-based anorexia.” The paradigm was used as a model for anorexia nervosa in humans and to induce similar clinical manifestations of the female athlete triad (28, 29, 46, 71). Despite the fact that the female athlete triad and anorexia nervosa are diagnosed more frequently in young women, only a few studies have used the female rat as the model.

In the activity-stress paradigm, rats are allowed free access to running wheels (22.5–24 h/day) and allotted ad libitum feeding only within a specified time (i.e., 1–1.5 h/day). Weight loss in running animals tends to be accelerated within the first few days because of lack of adaptation to imposed feeding schedules and increased running. Unless the animal learns to accommodate to the restricted feeding time, weight loss continues, anxiety-stress ulcers occur, and mortality rate is high (29, 87).

Design limitations of the classic activity-stress paradigm make it difficult to study the clinical manifestations of the female athlete triad. The goal of the present model was to modify the classic activity-stress paradigm to reduce rapid animal weight loss initially and maintain a stable body weight throughout the study, without mortality. The use of a voluntary wheel-running animal was important to the development of the model to duplicate voluntary human exercise. Additionally, rats were introduced to the running wheel at an early age to become acclimated and establish routine exercise patterns before the restricted-feeding phase. The effects of routine exercise on body composition and bone tissue were critical for establishing a rat model of the exercising female athlete. In contrast to the present model, female rats in the activity-stress paradigm are usually introduced to the running wheel just before the restricted-feeding regimen. As a result, these animals exhibit inconsistent and fluctuating patterns as they try to adjust to the changes, including 1) lower food intake within the first few days of initiating restricted feeding, which typically increases with time as they adapt to the feeding regime; 2) increased wheel running that peaks in 3–4 days and gradually decreases; and 3) rapid weight loss within a few days that results in an initial loss of estrus, with intermittent cycling throughout a typical 2- to 3-wk study period (12, 24, 43, 87).

Although irregular estrous patterns and out-of-sync cycling have been reported in exercising female rats with restricted feeding, the relationship of energy availability and ovulatory status has not been studied over the long term in the voluntary wheel running paradigm. The purpose of this study was to develop a model of the female athlete triad by examining the effects of long-term energy restriction in voluntary wheel-running female rats on estrous cycling, bone mineral content (BMC), and leptin levels.

MATERIALS AND METHODS

Research Design

The sample included 12 female Sprague-Dawley rats (34 days old) randomly divided into two experimental groups: 6 restrict-fed animals with free running wheel access and 6 ad libitum-fed animals with free running wheel access as controls after a 10-day acclimatization period. The investigation extended ~6 mo including the Baseline phases (95 days) of ad libitum feeding and free wheel access to establish patterns of dietary intake, body weight, estrous cycling, and wheel running behavior in all animals and the Treatment phase (94 days), which included the Weight Loss phase (38 days) followed by a 56-day Anestrous phase in six restrict-fed rats. The six control rats continued with ad libitum feeding and free wheel access throughout the Treatment period.

Animals

Approval to conduct this study was obtained from the Institutional Animal Care and Use Committee of the Texas Woman’s University. Rats were purchased from Charles River Laboratories (Wilmington, MA) and raised on standard laboratory rat chow (ad libitum) under sedentary conditions before arrival. Animals’ tails and cages were marked for identification, and all rats were housed individually in polycarbonate cages with stainless steel grid bottoms (Nalge, Rochester, NY). Animals had free access to an enclosed activity wheel (43-in. circumference or ~1 m (0.00109 km)), a diet bin, and filtered tap water. All rats were kept in an environmentally controlled room in the Animal Care Facility at Texas Woman’s University. The room was maintained at 21 ± 1°C with a constant 12:12-h light-dark cycle (light 0700 to 1900). Both groups of animals received the AIN-G (3.8 kcal/g body wt; during Baseline) and AIN-M (3.6 kcal/g body wt; during Treatment) rat chow formulas in pellet form (Dyets, Bethlehem, PA). Rats were injected with 50 mg/kg body wt pentobarbital to induce anesthesia and killed by decapitation after completion of the investigation.

Energy Availability

In general, the rat will consume food to meet its daily energy requirement (1, 48). Rats fed ad libitum may be unlike humans in that ad libitum-fed rats continue growing instead of achieving a relatively stable adult weight. Weight may not be regulated in rats, whereas growth rate may be. Age, temperature, reproductive status, and level of activity have all been shown to have a direct influence on the energy requirements of both female and male rats. In the female rat, the maintenance energy (ME) requirement is generally defined as that portion of the total energy requirement that is separate from the needs for growth, pregnancy, and lactation (14, 15). The ME requirement is usually expressed as energy required per unit of body weight, and typically animals fed at maintenance are in energy equilibrium or balance (17).

Because of expected changes in basal metabolic rate and a shift in energy balance among restrict-fed rats, the ME requirement was not calculated for animals in this study. To provide seamless reporting of data in control and restrict-fed animals during both Baseline and Treatment phases, estimation of energy for all animals was calculated with the energy availability formula (56, 59).

In this study, the terms energy intake, energy expenditure, and energy availability are used. Energy intake was calculated by multiplying the amount of food ingested in grams by the energy content of the food (either 3.6 or 3.8 kcal/g body wt). Energy expenditure for each animal in this study was estimated by determining the daily energy expended in wheel running. Exercise energy expenditure from daily wheel running was calculated with 21 kJ/kg body wt times kilometers run and then converted to calories (3, 4). The conversion of kilojoules to kilocalories is 4.1868 kJ/kcal. Thus daily wheel running energy expenditure was calculated as 5.0 kcal/kg body wt times kilometers run. This formula was used as the basis of the calculation of wheel running activity. Energy availability was calculated as energy intake minus exercise energy expenditure (56, 59). Restricted dietary intake and weight loss have been shown to lower metabolic rate in rats and humans (34, 62, 63, 89). In amenorrheic athletes,
resting metabolic rate was found to be 16% lower than in eumenor-303bic athletes (63). Therefore, to insure comparative energy data among controls and restrict-fed rats throughout the study, energy availability (energy intake – exercise energy expenditure) was determined instead of energy balance.

Restricted Feeding Regimen

Throughout Baseline, each rat exhibited individual patterns of diet intake, body weight, and voluntary wheel activity while maintaining a 4- or 5-day estrous cycle. Before Treatment, a 30% dietary restriction was calculated for each restrict-fed animal based on the previous 14-day ad libitum intake. Determination of diet restriction per animal was necessary because of individual growth rates and body weight, wheel running distance, and eating patterns that varied in amount of dietary intake across the estrous cycle. After desired weight loss was attained to initiate anestrus, the animals’ weight was regulated by a 3–5% incremental upward or downward dietary intake to maintain an average body weight at 1–2% below anestrous weight. This provided a margin of safety because of fluctuating daily wheel activity and consequential weight loss or gain. Upper limits of total weight loss throughout Treatment were set at a maximum of 30% for all animals. The restrict-fed rats followed a dietary regimen consisting of a daily allotment per animal fed once a day at 0900. The daily diet allotment was left in the feeding bin until consumed.

Data Collection

Data were collected daily from all animals during Baseline and Treatment with body weight, dietary intake, vaginal smears, and wheel exercise reported for each animal. At the end of the study, blood was collected from each animal during decapitation procedures, and individual samples were centrifuged and separated for determination of concentrations of serum estradiol and leptin. After death, selected femurs and tibias were cleaned and stored for bone mineral analysis. Left and right ovaries were excised, cleaned of excess fat and connective tissue, and weighed.

Daily procedures. Data collection of body weight, wheel exercise, and dietary intake were recorded between 0830 and 0930. Estrus was verified by vaginal smears, with daily monitoring between 0830 and 0930. Animal weight and diet intake were measured on a Sartorius (Mitter, Sunriver, OR), and after daily recording of activity counters were reset to zero. Wheel exercise data for each rat were reported as the number of kilometers, or distance run, per 24 h. Animals’ health and dietary intake were recorded between 0830 and 0900. The restrict-fed rats followed a dietary regimen consisting of a daily allotment per animal fed once a day at 0900. The daily diet allotment was left in the feeding bin until consumed.

Laboratory procedures. Laboratory assessments included 1) daily monitoring of estrous cycling measured by vaginal smear procedure, 2) radioimmunoassay (RIA) of serum estradiol and leptin collected at completion of study, and 2) poststudy assessment of BMC and BMD analyzed by dual-energy X-ray absorptiometry (DXA).

VAGINAL CYTOLOGY. Estrus was qualitatively verified by a vaginal lavage and smear technique with the use of a light-source microscope (Bausch & Lomb Optical, Rochester, NY), based on the protocol from prior research (54, 61). To identify each stage of the 4- or 5-day estrous cycle (estrus, proestrus, metestrus, diestrus), daily determination of vaginal cell type was monitored in each animal between 0930 and 1030 throughout the study. Characterization of individual estrous cycle phase was dependent on cell concentrations and assessed by percentage of leukocytes and nucleated or cornified epithelial cells in the smear. A vaginal smear profile was recorded daily for each animal, identifying cell types and ratio of each cell type from greater to lesser concentration. This ongoing record provided a chronicle of each animal’s pattern of reproductive cycling, from early peripubertal stages and first ovulatory cycles and continuing throughout adulthood. Anestrus was defined by acyclic and/or persistent diestrus vaginal cell patterns.

HORMONE ANALYSES. After decapitation, blood was drained from the body cavity into individual nonheparinized Vacutainer tubes. Blood samples were allowed to clot for 30 min and then centrifuged at 1,500 g for 10 min. Total serum estradiol was determined with a RIA kit (Coat-a-Count, Diagnostic Products, Los Angeles, CA). Results of sampled estradiol are expressed as SI units (pmol/l). Serum leptin concentration was measured by RIA at Linco Research (St. Charles, MO), and procedures for collection, handling, and processing of each sample were established before death and designated by laboratory protocol. Intra-assay coefficient of variations (CVs) for the estradiol and leptin assays were 5.3% and 3.3%, respectively.

BONE ANALYSES. After death and blood collection procedures, right and left femurs and tibias were excised, cleaned of excess fat and connective tissue, and stored in individually labeled scintillation vials. Left femurs and tibias were stored in Carson’s fixative for future analysis, and right femurs and tibias were stored in a saline solution at −20°C until assay. Measurements of femoral BMC and BMD were obtained by DXA (Lunar Radiation, Madison, WI). The DXA appendicular bone scans were measured by software specifically designed for small animals (DPX, Small Animal Software version 1.0, Lunar Radiation). To achieve a congruent beam of stable dual-energy radiation, the scanner used a K-edge filter (cerium) and an X-ray tube operated at 76.0 kVp (150 μA) in high-resolution mode with a source collimation (size of X-ray beam at the source) of 0.84 mm (fine). Throughout the study period, daily quality assurance tests were performed to ensure the effectiveness of the lights, beams, mechanics, and tissue value of the scanner.

The right femur and tibia were placed in a petri dish filled with distilled, deionized water to simulate soft tissue, and total femoral and tibial area for each sample were scanned. Intra-assay CVs in our lab were 0.285% and 0.850% for BMC and BMD, respectively. CV values reported in the literature range from 1.4% (BMC) to 0.7% (BMD) (74). The results are expressed in grams for BMC and grams per square centimeter for BMD.

Data analyses. In an experiment such as this, in which the aim is to produce pathological effects, these effects would be larger than the normal physiological range. Thus the effect size was calculated as 0.945. With a sample size of N1 = N2 = 6, and α = 0.05, power was calculated as 0.945.

Dependent and independent variables during Baseline and Treatment phases (dietary intake, body weight, wheel running distance, energy intake, energy expenditure, and energy availability) were examined by repeated-measures analysis of variance (ANOVA) in testing the changes among rats. Dietary intake and energy intake were controlled during the restrict-fed phase. Pearson’s correlation and Kendall’s τ were applied to determine relationships between dependent variables. Diet intake, body weight, wheel running distance, energy intake, energy expenditure, and energy availability were averaged across each 4- or 5-day estrous cycle and reported as means ± SE for each 20-day period of age, with significance determined at P < 0.05. After repeated-measures analysis, the data were examined in a series of steps, including tests of assumption of sphericity and normality. After 56 days of anestrus due to diet restriction and weight loss, analysis of response variables similar to criteria based on the female athlete triad were compared between groups (Mann-Whitney U statistic), including concentrations of serum estradiol and leptin,
femoral and tibial BMC and BMD, and final body weight and ovarian weight. All analyses were performed with SPSS 12.0 standard version.

**RESULTS**

**Baseline**

There were no significant differences in diet intake, body weight, or voluntary wheel running distance between groups at Baseline (30–129 days of age; Fig. 1). Diet intake paralleled animals’ increase in body weight. Wheel running distance peaked at 50–69 days of age, with gradual decrease until the end of Baseline. Daily patterns of diet intake and wheel running distance varied widely among individual rats, and a positive relationship was shown between wheel running distance and energy intake regardless of age ($R^2 = 0.479; P < 0.01$) (Fig. 2). After first estrus, each rat maintained normal turnover of leukocytic, nucleated, and cornified vaginal epithelial cells characteristic of each phase of the cycle.

In addition to age-related differences, all animals in this study exhibited regular estrus-related patterns with fluctuations in diet intake and wheel running that varied with the phase of the estrous cycle. Diet intake typically varied from 2 to 5 g throughout the 4- or 5-day cycle, with a greater amount consumed during the 24 h following estrus (estrus to metestrus) and a lower food intake observed during the 24 h following the day of proestrus (proestrus to estrus; $P < 0.01$). The range of wheel running across the 4- or 5-day cycle also varied. Daily wheel running was greatest during the 24 h following the day of proestrus (proestrus to estrus), and the least amount of wheel running occurred during the 24 h following the day of estrus (estrus to metestrus; $P < 0.01$). This pattern of greater and lesser wheel running across estrous phases continued throughout Baseline even though total wheel running distance decreased with animal age.

**Energy availability.** Although age-related changes in energy availability were noted, there were no significant differences in energy intake, energy expenditure, or energy availability between groups at Baseline (30–129 days of age; see Fig. 3). From age 30 to 129 days, mean energy intake increased from $60.3 \pm 2.1$ to $65.8 \pm 2.3$ kcal ($P < 0.01$), mean energy intake.
Rats lost body weight of 10% during Treatment (see Fig. 1). Restrict-fed diestrus, proestrus, or estrus (P phase of the cycle, with greater intake during metestrus vs. intake continued to fluctuate across the estrous cycle in control animals during Treatment. Among control animals, baseline diet intake during the last 2 wk ranged between 14.8 and 18.6 g/day, between 15 and 19.7 g/day during Weight Loss, and between 15.3 and 20.3 g/day during Anestrous. Diet intake continued to fluctuate across the estrous cycle in control animals, varying an average of 2–5 g/day depending on the phase of the cycle, with greater intake during metestrus vs. diestrus, proestrus, or estrus (P < 0.01).

Body weight. Control animals had a significant increase in body weight of 10% during Treatment (see Fig. 1). Restrict-fed rats lost ~28% in 6 wk of Treatment that also initiated and maintained anestrus. Weight loss ranged between 0.1 and 11 g/day, with a mean accumulated weight loss of 7.3% (6–9%; 1.1%/day) during week 1, 14% (12–17%; 1%/day) during week 2, 20% (17–27%; 0.8%/day) during week 3, 24% (21–28%; 0.5%/day) during week 4, 26% (23–30%; 0.4%/day) during week 5, and 28% (25–30%; 0.2%/day) during week 6. After the Treatment phases, the restrict-fed animals exhibited a mean final weight of 190.2 ± 10.7 g, which was 32% less than controls (279.7 ± 23.5 g).

Voluntary wheel running. An effect on wheel running was noted in both groups, with an overall decreasing trend (25%) from 8.3 ± 1.5 km/day (Baseline, age 110 days) to 6.2 ± 0.8 km/day (Anestrous; P = 0.016). In addition, dietary restriction did not result in greater wheel running, in contrast to the classic activity-stress paradigm. No significant differences were observed in wheel running distance of restrict-fed rats compared with controls at Baseline (9.5 ± 1.4 vs. 7.3 ± 1.6 km/day), Weight Loss (8.9 ± 1.0 vs. 6.9 ± 1.5 km/day), or Anestrous (6.9 ± 0.4 vs. 5.5 ± 0.9 km/day) (Fig. 1). Because no differences were shown in wheel running distance between animals, the ratio of absolute energy intake (in kcal) to wheel running distance (in km) was reduced in restrict-fed rats during Treatment. Before the Treatment phases (Baseline age 110 days), the ratio of energy intake to wheel running distance was similar in both groups of animals (controls = 8.6 kcal/km, restrict-fed = 7.3 kcal/km). The ratio of energy intake to wheel running distance in control rats and restrict-fed rats was 8.9 and 5.0 kcal/km, respectively, during Weight Loss and 11.3 and 5.4 kcal/km, respectively, during Anestrous (both P < 0.05). Finally, in contrast to Baseline, there was no correlation between dietary intake and wheel running in restrict-fed animals.

Energy availability. Restrict-fed rats’ mean energy intake compared with control rats during the Weight Loss phase (Fig. 3) was 44.8 ± 2.5 vs. 61.7 ± 2.5 kcal (P = 0.002), mean energy expenditure was 10.2 ± 1.4 vs. 8.7 ± 1.8 kcal (P = 0.016), and mean energy availability was 34.4 ± 2.0 vs. 52.5 ± 1.3 kcal (P = 0.002). Restrict-fed rats’ mean energy intake compared with control rats during Anestrous was 37.5 ± 0.9 vs. 62.2 ± 2.4 kcal (P = 0.002), mean energy expenditure was 6.5 ± 0.4 vs. 7.3 ± 1.1 kcal (P = 0.016), and mean energy availability was 31.0 ± 0.8 vs. 55.4 ± 1.5 kcal (P = 0.002). Although energy intake decreased in restrict-fed rats, energy expenditure did not change and was similar to controls during Weight Loss and Anestrous phases. Thus the restrict-fed animals showed a significant decrease in energy availability during both Treatment phases (P < 0.01).

Estrous cycling. Estrous cycling continued with ad libitum-fed control animals in established 4- or 5-day patterns throughout Treatment. Cessation of normal estrous cycling and acyclic vaginal smears [void of nucleated (proestrus) cells] occurred in all restrict-fed animals after 22–26 days of restricted feeding and a weight loss of 19–28% (mean = 24%). Within the second week of dietary restriction (2–3 estrous cycles), animals exhibited sequential aberrant cycling patterns including an increase in the number of days per cycle; a decrease in number of vaginal cells, followed by irregular and out-of-sync cycling patterns with limited nucleated (proestrus) cells; and finally anestrus. Persistent diestrus patterns were not shown in all animals until days 26–32 (mean = 29.8 ± 3.2 days) of restricted feeding. Persistent diestrus vaginal cell patterns representing anestrus continued throughout the study. Anestrus
was maintained in five restrict-fed animals for the duration of
the study, with one animal spontaneously resuming estrous
cycling after 44 days of persistent diestrus and returning to a
6-day cycle until study completion.

Leptin. At the end of the study, serum leptin levels were
significantly decreased in restrict-fed animals compared with
control animals \((P < 0.002)\), with four animals in the restrict-
fed group exhibiting concentrations less than the minimal
detectable value \((0.5 \text{ ng/ml})\) (see Table 1).

Estradiol and ovarian weight. Compared with control rats,
serum estradiol was 62% lower \((P < 0.002)\) in restrict-fed rats,
with a limited range of estradiol concentration compared with
control rats (see Table 1). Additionally, ovaries of the restrict-
fed rats were atrophied after 3 mo of caloric restriction and 2
mo of anestrus, exhibiting a weight difference of 57% com-
pared with control animals.

Bone data. A significant difference between groups was
shown for both femoral BMC \((P = 0.041)\) and tibial BMC
\((P = 0.05)\). Femoral \((P = 0.065)\) and tibial BMD \((P = 0.296)\)
were not significantly different between the two groups (see
Table 1).

DISCUSSION

The purpose of this study was to develop a rat model
representing the female athlete triad. Similar criteria were
designated in this model to characterize a profile of the energy-
deficient and amenorrheic athlete. In contrast to the classic
activity-stress paradigm, specific objectives included use of a
young adult female rat with lifelong voluntary access to an
exercise wheel; controlled and progressive weight loss; nonin-
termittent anestrus; and stabilized lower body weight without
continued weight loss or mortality. The study objectives were
achieved and included the survival of all animals.

Energy Availability

Energy availability is critical for ovulation in female rats \((5,
15, 20, 31, 81)\). Energy availability is dependent on energy
intake and energy expenditure. If energy intake is inadequate,
energy availability is compromised, resulting in a lack of
 calories to maintain estrous cycling.

One of the more significant findings of this study was that
the correlation of energy intake and wheel running distance
was not maintained by the restrict-fed rats during Treatment. In Baseline, energy intake correlated positively with voluntary wheel running activity, i.e., the greater the running distance, the greater the dietary intake in ovulating ad libitum-fed rats. These results were similar to those of other studies of ad libitum-fed wheel-running rats. Although energy intake has typically been shown to be greater in wheel-running vs. sedentary ad libitum-fed female rats (4, 35, 44), only a few studies have reported a positive correlation between energy intake and wheel running distance in individual animals (42, 77). Clearly, wheel running and energy intake in ad libitum-fed and ovulating female rats are correlated positively, but it is yet to be determined whether there is a causal effect of energy intake on wheel running distance or wheel running distance on energy intake. Energy studies by Anantharaman-Barr and Decombaz (3, 4) on the effect of wheel running on positive energy balance in ad libitum-fed rats “demonstrated the precision with which the female rat regulates long-term energy balance. Over a 6-mo period, weight gain was not affected by running since the active rats [wheel running] increased food consumption to make up for the energy cost of running.”

Patterns of greater wheel running distances coupled with greater energy intake and estrus-related fluctuations in energy intake and wheel running persisted in both the growing and the mature ovulating animal. Although overall running distance was positively associated with greater consumption of diet, estrus-related fluctuations in energy intake and wheel running typically exhibited an inverse relationship, with greater energy consumed during days of lower wheel exercise. Previous research showed that female rats exposed to ad libitum feeding and voluntary wheel access exhibit both circadian and diurnal rhythms, with fluctuating levels of wheel activity and energy intake that vary throughout the light-dark cycle as well as the 4- or 5-day estrous cycle (4, 44, 78, 84). In light of the observation that both wheel running activity and energy intake are regulated throughout estrus, several investigators have suggested that the female rat exists in a state of oscillating energy balance that is influenced by fluctuating levels of ovarian hormones (4, 42).

Thus, during Baseline, rats that expended more wheel running energy compensated with greater energy intake, allowing for greater available energy to sustain growth and ovulation. This voluntary and tightly controlled compensatory mechanism of energy regulation resulting in greater energy availability was a hallmark of the wheel-running female rat. Interestingly, the restrict-fed rats did not exhibit this same biological response to match wheel running energy expenditure to energy intake. Dietary restriction did not automatically cause a reduction in voluntary wheel running distance to compensate for the decrease in energy intake. These findings in the restrict-fed animals are similar to human data that indicate a rather loose coupling between energy intake and energy expenditure, with findings of “no strong biological drive to match energy intake to activity-induced energy expenditure” (79) and “physical activity does not automatically generate an increase in the drive to eat that would compensate for the energy expended” (12).

Based on energy balance studies in the ovulating female rat, the tightly controlled compensatory mechanism of energy availability may have been altered, leading to a downregulation of the hypothalamic-pituitary-ovarian axis and ovarian hormones (4, 31, 42, 82, 83).

In the development of this model, critical components necessary to simulate the female athlete triad included anovulation and stabilized body weight to ensure animal survival. The final phase of Treatment (Anestrous) was characterized by acyclical and persistent diestrous patterns; an age-related decrease in wheel running distance similar to ad libitum-fed control animals; a narrow range of wheel running with minimal fluctuation; and stabilized body weights, without further weight loss or gain. An individual energy intake based on body weight, or metabolic set point, has been theorized to be necessary to support ovulation as well as induce and/or maintain anestrus (82, 83). From previous animal and human research, onset of anovulation due to energy-induced weight loss has been shown to be approximately 10–25% of initial weight in women with amenorrhea (39, 47, 81, 85, 86) and 20–30% in female rats (25, 29, 49, 87). In this study, once rats reached anestrus after a 30% weight loss, a specific energy intake based on body weight was continued to stabilize body weight and maintain anestrus throughout the study.

Leptin

Weight loss and reduced body fat are also associated with decreased concentrations of serum leptin. Leptin is secreted by adipocytes, and therefore serum and tissue concentrations are highly correlated with body adiposity and fat mass (21, 60). Secretion of leptin has also been implicated as a metabolic signal in the balance of energy, impacting on regulation of food intake, body weight, and the exchange and deposition of adipose tissue (18, 36). Reports show that profound fluctuations in leptin occur before changes in body adiposity due to a response to fasting, dietary restriction, refeeding after dietary restriction, and overfeeding (38, 50, 88). Furthermore, a positive association between estrogen and leptin concentrations has been shown in female rats. Shimizu et al. (73) reported that after ovariectomy (OVX) rats showed a decrease in obese gene expression in adipose tissue coupled with a reduction in serum leptin, and with administration of estradiol the effect of OVX on obese gene expression and serum leptin was reversed.

In this study, serum leptin levels were markedly reduced in restrict-fed animals compared with ad libitum-fed control

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<td>Femoral BMC, g</td>
<td>Restrict-fed</td>
<td>0.307</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.360</td>
<td>0.04</td>
</tr>
<tr>
<td>Tibial BMD, g/cm²</td>
<td>Restrict-fed</td>
<td>0.192</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.201</td>
<td>0.11</td>
</tr>
<tr>
<td>Tibial BMC, g</td>
<td>Restrict-fed</td>
<td>0.221</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.260</td>
<td>0.03</td>
</tr>
</tbody>
</table>

n = 6 each for restrict-fed and control rats. BMD, bone mineral density; BMC, bone mineral content. Leptin and estradiol levels, ovarian weight, and femoral and tibial BMC were significantly different in restrict-fed vs. control rats (P < 0.05).
animals, with four restrict-fed animals exhibiting concentrations at less than the minimal detectable value. Similar reports of decreased concentrations of leptin have been reported in both animal and human studies of weight loss and in subjects exhibiting a lower BMI and reduced adipose tissue (21, 60, 69).

**Estradiol/Ovaries**

The wide variation in estradiol concentration in the control animals is within the normal range for ovulating rats and reflects fluctuation across the estrous cycle. Comparative levels of estradiol have been reported in other ovulating ad libitum-fed rats with access to running wheels and have also been shown to be similar in sedentary animals (16, 29).

Serum estradiol was significantly lower in restrict-fed rats compared with control rats, and the range of estradiol concentration was substantially smaller compared with the ad libitum-fed animals. Ovaries of the restrict-fed rats exhibited atrophy after 2 mo of anestrus, with a significantly smaller weight than control rats.

Previous studies of female rats that have been diet restricted and/or animals with weight loss report lower concentrations of estradiol and ovarian weight similar to ovariectomized rats (19, 25, 62). The importance of using an ovary-intact animal with reduced concentrations of estradiol was essential to simulate conditions of the female athlete triad.

**Bone**

The combined effect of diet restriction, weight loss, and anestrus on bone has not been studied in the ovary-intact female rat with access to voluntary wheel running. As a result, comparative data are unavailable for this rat model. Alternatively, bone-related data that examine one or more features of this model have been shown in both sedentary and exercised animals exposed to wheel running, and although a direct comparison of results cannot be determined, similar characteristics of bone-related changes due to diet restriction, weight loss and low body weight, and estrogen depletion can be inferred.

Both endurance exercise and voluntary wheel running have produced a positive effect on bone mass in rats. Although exercising animals tend to gain weight more slowly and weigh less than sedentary rats, diet intakes are greater and animals exhibit increased bone mass with running. The anabolic effects of voluntary wheel running in ad libitum-fed rats have been shown to have a positive and significant impact on multiple axial and appendicular skeletal sites (9, 35, 40, 66, 90, 96). Diet restriction has been shown to impact bone growth and bone turnover in female and male rats of all ages, with a decrease in osteoblastic activity and bone formation, a reduction in bone turnover, and diminished bone mass (25, 67, 75). Restrictive diets in young and aging rats result in both a generalized detrimental effect throughout the skeleton as well as an altered bone response that is site specific and influenced by animal age.

Similar to diet restriction, reduced estrogen and a lack of ovulation can have detrimental effects on bone mass. The effects of low estrogen on bone have been studied extensively in anovulation and in the OVX rat as a model for bone loss that occurs in postmenopausal women (23, 33, 37, 45, 72, 77, 93). OVX-induced bone loss due to estrogen deficiency in the female rat shares a wide range of similar characteristics with estrogen deficiency-induced bone loss in women, including an increased rate of bone turnover, with resorption exceeding formation, an initial rapid phase of bone loss followed by a much slower phase, a greater loss of cancellous than cortical bone tissue, and a decrease in intestinal absorption of calcium.

In the present investigation, decreased energy intake was followed by weight loss and anestrus, with significantly lower estradiol concentration. The combined effects of energy restriction and a lack of adequate estrogen may have a greater impact on bone than a deficiency of either by itself. In addition, energy restriction is typically followed by weight loss and a lower body weight. Body weight has consistently been shown to be a reliable determinant of bone mass, and a lower body weight is associated with lower bone mass. Rat studies show that in ovary-intact and OVX animals the combined effects of diet restriction and weight loss have a greater effect on bone loss than estrogen deficiency alone (25).

Restrict-fed rats continued wheel running as much as control rats in this study throughout Baseline and Treatment phases, but the beneficial effect of wheel exercise on bone was compromised. As shown by previous research, the lower bone mass exhibited by the restrict-fed animals may have been the result of one or more factors, i.e., the reduction in energy intake, a lower body weight due to a 25–30% weight loss, the estrogen depletion effects of anestrus (−60 days), or the combined effect of all three factors.

The specific objectives in developing this animal model of the female athlete triad were obtained. The critical components necessary for the success of this model include the following: 1) use of female Sprague-Dawley rats because of their consistency as runners; 2) early access to voluntary wheel running to ensure stabilization of energy availability during growth and development; 3) ad libitum feeding during Baseline to establish patterns of energy intake, body weight, and wheel running distance; 4) daily vaginal smears to monitor consistent and/or aberrant patterns of estrous cycling; 5) initiation of the restricted-feeding regimen in young adult rats after stabilization of growth rate; 6) restricted feeding based on individual 70% dietary intake at Baseline; 7) feeding animals once a day in the morning; 8) incremental 3–5% increase or decrease in dietary intake to ensure slow and progressive weight loss during the Weight Loss phase and stabilization of body weight during the Anestrous phase.

The duration for the development of the present animal model may be a potential problem and/or limitation. Because of age-related patterns in diet intake, body weight, and wheel running distance, the initial age of the animal and the length of the study should be a primary consideration in future research. Consistency of results and effectiveness of the model are dependent on the use of female rats of similar age and species that are exposed to like conditions. Exposure to the running wheel at an early age during the Baseline phase is essential in establishing the effects of routine exercise on body composition and bone tissue similar to the young exercising female. Additionally, individual patterns of ad libitum diet intake, body weight, and voluntary wheel running would potentially impact bone mass in the young adult and mature rat. Ages of the animals during the Treatment phase were equivalent to the young adult, extending beyond primary growth stages and pubertal development—developmental factors that are critical to bone accrual.
We believe that the “energy drain” hypothesis can be used as the paradigm to develop an animal model of the female athlete triad. The female athlete triad may be a specific expression of a general mammalian function intended for survival of the species, e.g., the energy or metabolic cost of normal menstrual cycling is expendable during high energy drain and may serve as an adaptive response to preserve other nonexpendable tissues, an adaptive response that is similar to natural environmental stresses due to famine or seasonal shortages in food supplies (53, 80, 94).

The results from this study suggest that voluntary exercise does not negatively impact normal estrous cycling in the young ad libitum-fed rat, regardless of wheel running distance. Instead, energy expenditure was greater and maintained the caloric cost of estrous cycling, whereas in the restrict-fed animals, a decreased ratio of energy intake to wheel running energy expenditure resulted in decreased energy availability, leading to weight loss, anestrus, and significant decreases in estradiol, leptin, and BMC.

REFERENCES
21. Dempster DW, Birchman R, Xu R, Lindsay R, Shen V. Temporal changes in cancellous bone structure of rats immediately after ovariec-


