Effect of the estrous cycle and surgical ovariectomy on energy balance, fuel utilization, and physical activity in lean and obese female rats.

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Running Head: Obesity, Estrous Cycle & Energy Balance

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ABSTRACT:
This study presents an in depth analysis of the effects of obesity on energy balance and fuel utilization in adult female rats, over the estrous cycle and immediately after surgical ovariectomy (OVX), to model pre- and post-menopausal states, respectively. Female Wistar rats were fed a high fat (46%) diet for 16 weeks to produce mature lean and obese animals. Daily vaginal lavages were performed to identify stage of estrous, while monitoring energy intake (EI), total energy expenditure (TEE), and fuel utilization, in a multi-chamber indirect calorimeter with activity monitored by infrared beam breaks. Metabolic monitoring studies were repeated during the 3-week period of rapid OVX-induced weight gain. Component analysis of TEE was performed to determine both the non-resting and resting portions of energy expenditure. Obesity was associated with a greater fluctuation in energy balance across the estrous cycle. Cycling obese rats were less active, expended more energy per movement, and oxidized more carbohydrate than lean rats. The changes in energy balance over the cycle in both lean and obese rats were driven by changes in EI. Finally, OVX induced a large positive energy imbalance in both obese and lean rats. This resulted primarily from an increase in EI in both groups, with little change TEE following OVX. These observations reveal a dominant effect of obesity on energy balance, fuel utilization, and activity levels in cycling rats, which has implications for studies focused on obesity and energy balance in female rodents.

KEY WORDS:
Energy intake, energy expenditure, indirect calorimetry, spontaneous activity, menopause
INTRODUCTION:

Perturbations in the regulation of energy balance and fuel utilization underlie the development, progression, and recurrence of obesity (31, 36). This dysregulation in energy homeostasis has been linked to the development of many obesity-related co-morbidities, including diabetes (2, 24), cardiovascular disease (24), and certain types of cancer (11, 42, 45). Understanding the impact of this dysregulation on obesity and its co-morbidities has led to a greater need for accurate measurements of energy balance and fuel utilization in relevant disease models. This has been challenging in preclinical studies. Although measuring food intake is standard practice, simultaneous measurements of energy expenditure and fuel utilization that provide a more complete picture of energy balance are less common. Measuring these parameters becomes especially important for studies in females because energy balance and fuel utilization are significantly affected by the estrous cycle (4, 6, 10, 25).

Many estrous cycle-related effects have been linked to the effects of estrogens on both the central nervous system and peripheral tissues (4-6, 15, 23, 37, 46, 52). Rodents typically cycle over 4 to 5 days, and the phases of this cycle are commonly classified by histologic changes in vaginal cytology, which are roughly divided into days: diestrus 1 (D1), diestrus 2/3 (D2), proestrus (P), and estrus (E) (47). We and others have reported that estradiol begins to rise in D2, peaks in P, and rapidly drops to negligible levels in E (4, 47). The latency of estrogen’s suppression of food intake is approximately 12-24 hours and has been attributed to the time course required to alter gene expression in both central and peripheral tissues (6). Consequently, food intake is generally elevated in D1 and D2, declines in P, and reaches a nadir in E (17). Less is known about the concomitant changes in energy expenditure and fuel utilization in rodents, but it is often assumed that the changes in physical activity observed across the estrous cycle are reflective of changes in total energy expenditure (TEE). Female rats, ferrets, hamsters, and cows have all shown increased activity during E, whereas similar
studies in monkeys did not report changes in activity over the cycle (3, 9, 16, 17, 38). Overall, the inference from these studies is that the rise in estrogens that occurs in D and early P leads to decreased EI, increased TEE, a negative energy imbalance, and acute weight loss. In contrast, the loss of estrogens, as occurs with surgical ovariectomy (OVX), leads to a persistent positive energy imbalance and weight gain (23, 30, 33).

The cyclical changes in body weight over the cycle (D2>P>D1>E) provide evidence for coordinated and opposing changes in EI and TEE during the estrous cycle. However, the literature lacks confirmation with a comprehensive examination of energy balance, with simultaneous measurements of EI and TEE at each stage of the estrous cycle. Furthermore, it is not known whether obesity alters such cycle-related changes in energy balance, nor have the effects of OVX on energy balance been fully characterized. Thus, the purpose of the present study was to examine the impact of obesity on both cycle-related and OVX-induced changes in energy balance and fuel utilization. We hypothesized that changes in metabolism across the cycle, and following OVX, would be blunted in the obese when compared to lean rats. These data fill a gap in metabolic research on female rodents, and provide the foundation for studying the impact of energy balance and fuel utilization on obesity and its related comorbidities.

EXPERIMENTAL PROCEDURES:

Experimental Design: Female Wistar rats (126–165 g; 5 weeks of age) were purchased from Charles River Laboratories (Charles River Laboratories, Wilmington, MA) and were housed in the University of Colorado Denver Center for Comparative Medicine and the Center for Human Nutrition Satellite Animal Facility (22–24°C; 12:12 h light-dark cycle) with free access to water. All procedures were approved by the institutional animal care and use committee. Obese and lean rats were identified by their differential response to a diet high in fat (12, 33). Briefly, rats were individually housed in wire bottom metabolic cages that limit activity (relative to group
housed animals in polycarbonate cages), and were given free access to a high fat diet (HF; 46% kcal fat) (Research Diets, New Brunswick, NJ; RD# D12344). Rats were ranked by their rate of weight gain in this obesogenic environment from 10 to 18 weeks of age. Those in the top tertile of weight gain were classified as obese (n = 25), and those in the lower tertile as lean (n= 23). Rats from the middle tertile were not used for this study. For all experiments, fully mature, adult lean and obese rats were studied between the ages of 20 and 30 weeks, a period characterized by growth plateau (35), with *ad libitum* access to this HF diet. Body composition was determined by dual-energy X-ray absorptiometry (DXA) using the Lunar DPX-IQ (GE Lunar Corp., Madison, WI), with Lunar’s Small Animal Software Version 1.0, or by quantitative magnetic resonance (qMRI; Echo MRI Whole Body Composition Analyzer; Echo Medical Systems, Houston, TX).

**Identification of stage of estrous cycle:** Vaginal lavages were performed daily, approximately 5 hours prior to the onset of the dark cycle, to identify each animal’s stage of the estrous cycle. Approximately 200 μL of PBS + 0.2% Brij 35 detergent (Sigma-Aldrich, St. Louis, MO) was used for each procedure and the unstained samples were examined under a light microscope. Stage of cycle was assigned using the following criteria, as previously described (32, 47): Proestrus (P) – predominately nucleated epithelial cells in the absence of leukocytes; Estrus (E) – sheets of nonnucleated squamous cornified cells in the absence of leukocytes; Diestrus 1 (D1) – equal distribution of leukocytes, cornified, and nucleated epithelial cells; and Diestrus 2 (D2) – a mixture of epithelial and leukocytes, with a predominance of leukocytes, following Diestrus 1. Cycle phases were assigned to the 24-hour period prior to the lavage. We had a small number of animals that failed to cycle, or did not cycle consistently. It is unclear from our analysis whether this could be attributed to either the age or adiposity/weight of the animals, or timing of the vaginal lavages (7, 19). Non-cycling animals were excluded from the pre-OVX phase of the study and for this reason not all post-OVX data points have a corresponding pre-OVX
Metabolic Monitoring System: A metabolic monitoring system (Columbus Instruments, Columbus, OH) was used to assess energy balance, fuel utilization, and activity in rats both in the pre-menopausal phase, and following surgical ovariectomy (OVX). This multichamber indirect calorimetry system allows for the continuous monitoring of up to eight rats, obtaining measurements of oxygen consumption ($vO_2$) and carbon dioxide production ($vCO_2$) from each chamber every 16 minutes (27, 36). The chambers also allow for the collection of daily urine, feces, and food spillage. Animals were removed from their cages approximately 5 hours prior to the onset of the dark cycle each day while the cages were cleaned and vaginal lavages were performed.

Animals were placed in the metabolic cages at least two days prior to the collection of metabolic data to allow for acclimatization to the new environment. Metabolic measurements were obtained for each animal across at least two full estrous cycles prior to ovariectomy or SHAM surgery. Ovariectomies were performed by placing rats under isoflurane anesthesia and surgically removing the ovaries using an intra-abdominal approach. Following surgery, animals were allowed to recover for 5 days, at which time they were returned to the metabolic monitoring cages. For each animal, the first day of calorimetry following OVX, during which intake and expenditure were equal (± 3 kcal), was identified as the energy balance day (OVX-EB). On average, this was the second day in the metabolic monitoring system, or 7 days post-OVX. The animals were then monitored over the subsequent 2 weeks of OVX-induced weight gain (OVX-Gain). Urine was collected over each 24-hour period for the estimation of protein disappearance.
**Energy Expenditure:** Metabolic rate (MR) was calculated from gas exchange measurements acquired every 16 minutes over the 24-hour period using the Weir equation \( MR = 3.941 \cdot vO_2 + 1.106 \cdot vCO_2 - 2.17 \cdot N \), where \( N \) represents urinary nitrogen) (53). In addition, the data were used to acquire both resting energy expenditure (REE) and non-resting energy expenditure (NREE). Resting metabolic rate (RMR, cal/min) was estimated as an average metabolic rate over a 1-hour period occurring in the latter part of the light cycle, as previously described (35), and was extrapolated throughout 24 hours to obtain resting energy expenditure (REE). For each animal, the 1-hour period was selected during a time in which metabolic rate was at a nadir and no activity was detected. It is important to note that in these *ad libitum* fed animals, RMR does not necessarily equate to basal metabolic rate. Animals were not fasted when this measurement was acquired. As such, the REE calculation may include some component of the thermic effect of food, particularly in the OVX animals that are in a large positive energy imbalance. Non-resting energy expenditure (NREE) was calculated as the difference between TEE and REE. All data is expressed as kcal/day.

**Calculations of Fuel Utilization:** Respiratory exchange ratio (RER) was calculated as the ratio of \( CO_2 \) produced to \( O_2 \) consumed \( (vCO_2/vO_2) \). Estimates of whole body substrate oxidation were calculated from \( vO_2 \) (L/min) and \( vCO_2 \) (L/min) measurements when in energy balance, and from measurements of urinary nitrogen \( (N; g/min) \), using derivations of Weir’s equation as follows:

- Carbohydrate disappearance (g/min) = \((4.57 \cdot vCO_2) - (3.23 \cdot vO_2) - (2.6 \cdot N)\)
- Lipid disappearance (g/min) = \((1.69 \cdot vO_2) - (1.69 \cdot vCO_2) - (2.03 \cdot N)\)
- Protein disappearance (g/min) = \(6.25 \cdot N\)

While in energy balance, calculations of substrate disappearance are a good reflection of substrate oxidation. When out of energy balance, however, it is critical to also consider the relative impact of metabolite interconversions. Ketogenesis, lipogenesis, and gluconeogenesis,
can affect substrate disappearance such that it becomes less reflective of oxidation alone, and this must be considered in the interpretation of substrate disappearance data.

**Activity Measurements:** Each metabolic cage was equipped with an Opto-Max animal activity meter (Columbus Instruments, Columbus, OH). This consists of a 1-dimensional series of infrared beams that, when broken by the animals’ movement, allow for the measurement of total activity, as well as both ambulatory and non-ambulatory activity. Both ambulatory and total activities were monitored continuously over the 24-hour period, and subtraction of ambulatory counts from total counts was used to assess stereotypic activities such as grooming, scratching, feeding, and other non-ambulatory activities.

**Plasma and Urine Measurements:** Tail vein blood was collected on D2 of the estrous cycle during the week prior to OVX, and again at the time of sacrifice. In all cases, blood draws were made during the latter part of the light cycle, and plasma was isolated and stored at -80 °C until analyzed. Estradiol was measured by enzyme immunoassay (Alpco Diagnostics, Salem, NH). Colorimetric assays were used to measure plasma free fatty acids (Wako Chemicals USA, Richmond, VA), glucose, triglycerides, and total cholesterol (#TR15421, TR22321, and TR13521, respectively; Thermo Fisher Scientific, Waltham, MA). Urinary nitrogen was estimated from measurements of urea and creatine in 24-hour urine collections, as previously described (34, 36).

**Statistical analysis:** Data were analyzed by two-way ANOVA (obesity, cycle day, and their interaction), with planned comparisons between lean and obese groups, using SPSS software version 17.0. Cycling SHAM and pre-OVX data were combined, as there were no differences between the two groups in any of the measured parameters. For some parameters, the data
were further analyzed by ANCOVA to adjust for the variation due to specified covariates. Significance was set at \( p < 0.05 \).

**RESULTS:**

*Morphometrics:* Morphometric characteristics of intact (cycling) and OVX animals are shown in Table 1. When entering in to the study period, obese rats had a higher percentage of body fat \( (p < 0.001) \) and weighed about 20% more than lean rats \( (p < 0.001) \). This greater body weight was due to both a 35% higher fat mass, and 12% higher fat free mass \( (p < 0.001 \) for both).

In response to OVX surgery both lean and obese animals experienced a brief period of weight loss. By five days post-OVX, all animals began to gain weight. Using this experimental paradigm we have previously shown that OVX induces rapid weight gain for approximately 3 weeks, after which the rate of weight gain returns to SHAM or pre-OVX levels \( (33) \). In the current study, animals were studied during this critical three week window of rapid weight gain in order to determine if OVX-induced weight gain is due to changes in energy intake, expenditure or both. Although we did not follow the animals long enough for their total body weight to significantly surpass their pre-OVX weight, we did see a significant increase in the rate of weight gain in both the lean and obese groups following OVX \( (p < 0.001) \).

*Energy Balance:* Daily energy intake \( (EI) \), total energy expenditure \( (TEE) \), and energy balance \( (EB) \) (calculated as \( EI – TEE \) throughout the estrous cycle, and following OVX are depicted in Figure 1. Both obese and lean rats exhibited a positive energy imbalance \( (EI > TEE) \) during D1, D2, and P, and shifted into negative EB during E \( (EI < TEE) \) (Figure 1a). The pattern of this cycle-dependent change in EB was significantly different in lean and obese rats \( (p = 0.021) \). Most notably, the obese had a much wider range of EB across the cycle (from +17 to -10 kcal/day) than the lean (+13 to -3 kcal/day). On D1 and D2, both lean and obese rats exhibited
a similar positive energy imbalance. As the animals progressed to P, obese rats maintained this level of positive imbalance, while the caloric excess in the lean rats decreased significantly (p=0.046). Finally, during E, both groups exhibited a negative energy imbalance, and this imbalance was significantly greater in the obese (p=0.047). Rats that underwent OVX were examined in energy balance, prior to any OVX-induced weight gain (OVX-EB). This allowed us to examine the impact of OVX on EB, without the complication that increased food intake and increased body weight would impart on these measurements during OVX-induced weight gain. Following OVX, both lean and obese animals were in significant positive imbalance (27 and 25 kcal/day excess, respectively), with no difference between groups.

**Energy Intake (EI):** The fluctuations in energy balance through the cycle were driven primarily by changes in energy intake (Figure 1b). In both lean and obese cycling rats, EI varied significantly according to day of estrous cycle (p<0.001), with intake reaching its peak during diestrus (D1 in lean rats; D2 in obese), and its lowest during E. Obese rats had a higher EI during D2 and P (p<0.05 for both) and a wider range of mean intakes than leans across the cycle (25 vs. 14 kcal/day). Following OVX surgery, EI increased significantly in both groups (p<0.001), reaching levels beyond the highest intake in cycling animals. Given that across the estrous cycle, the average EI tended to be higher in the obese animals (p=0.096), and that obese and lean did not differ in their intake following OVX, it would appear that the impact of OVX on altering food intake may be more substantial in the lean, than in the obese. While this difference did not reach statistical significance, there was a trend for a greater increase in intake from pre- to post-OVX in the lean (p=0.061).

**Total Energy Expenditure (TEE):** The cycle itself had no impact on TEE; however, cycling obese rats expended more energy than their lean counterparts (Figure 1c, p<0.001). This can be explained primarily by differences between the two groups on D1 (p=0.037), D2 (p=0.003)
and E (p=0.034). In general, fluctuations in TEE throughout the cycle were smaller (~4 kcal/day range) than the fluctuation in EI (~25 kcal/day range). Likewise, the impact of OVX on TEE (assessed in OVX-EB rats) was negligible, while EI was increased by more than 15 kcal/day in response to OVX.

**Component Analysis of TEE – REE and NREE:** Total energy expenditure was separated into its resting (REE, Figure 2a) and non-resting (NREE, Figure 2b) components. As expected given their higher body weight and lean body mass, REE was greater in obese rats across the cycle (p<0.001). Differences in REE between lean and obese animals are often attributed to differences in body composition; however, the higher REE in the obese remained significant after statistically adjusting for fat free mass (p<0.001). REE did not change significantly over the days of the estrous cycle, although REE was higher during E, when compared to P (p=0.043). Following OVX, REE increased in the lean to match REE in the obese group. In contrast to REE, NREE was similar for lean and obese rats across the estrous cycle, and decreased following OVX in both groups (p=0.002, Figure 2b).

Given the larger body weight of obese rats, a similar NREE in lean and obese groups required that activity levels be lower in the obese. This was found to be the case, whether examined as total activity (Figure 3a, p<0.001), or when separated into ambulatory activity or nonambulatory activity (p<0.001 for both, data not shown). Obese rats were approximately 40% less active than lean animals across the entire cycle. In both groups, activity also varied across the estrous cycle. When compared to D1, D2, and P, activity during estrus was 14% higher in the lean (p=0.027) and 20% higher in obese (p<0.001). Following OVX, obese rats remained less active than the lean, and activity in both groups decreased to levels below those observed at across the estrous cycle.
The fact that lean rats were more active than the obese, with no difference in NREE would suggest that the energetic cost of each activity movement is greater in the obese than in the lean. Further examination of the relationship between NREE and activity supports this notion. As shown in Figure 3b, NREE positively correlated with activity in both the lean (R=0.413, p<0.001) and the obese (R=0.510, p<0.001). Additionally, the slope of the regression curve for this relationship was significantly greater in the obese than in the lean (slope 0.0006 vs. 0.0001, p<0.001). Not only does this indicate that the obese have a higher energetic cost for each unit of movement, it also demonstrates that this energetic cost increases precipitously as overall activity increase.

Non-Protein Respiratory Exchange Ratio (NP-RER): Calculations of NP-RER are used to estimate which fuels are being oxidized, with 1.0 representing carbohydrate oxidation and 0.7 representing fat oxidation. NP-RER changed significantly over the estrous cycle (Figure 4a; p<0.001), reaching its highest values during D1/D2 (enhanced carbohydrate oxidation) and lowest during E (enhanced fat oxidation). While the pattern of 24-hour NP-RER was similar in lean and obese animals, the magnitude of the changes were greater in the obese rats, with a trend for higher NP-RER in the obese on D2 (p=0.081) and significantly higher NP-RER on P (p=0.031). In general, NP-RER varied primarily as a function of EB (R=0.744, p<0.001), but it was also inversely related to activity levels (R= -0.211, p<0.01). Together, EB and activity levels explained 52% of the variation in NP-RER over the cycle. Notably, NP-RER was similar for lean and obese animals on E, even though the obese rats exhibited a more substantial negative energy imbalance. This provides indirect evidence that lean animals are more prone and/or have a greater capacity to utilize fat under conditions, such as negative EB, that favor the oxidation of this substrate. Post-OVX, 24-hour NP-RER increased in both lean and obese animals, with no differences between the two groups. When NP-RER was examined during both the dark and light cycles, a similar pattern was observed in both lean and obese animals.
**Substrate Disappearance:** When in energy balance, substrate disappearance data provide a good reflection of substrate oxidation. Substrate disappearance was averaged over the entire cycle, during which the net energy imbalance was minor (~11 kcal/day) in both lean and obese groups. Given this slight energy imbalance, it is important to make the distinction between substrate disappearance and oxidation, as there remains a possibility of metabolite interconversions and/or net retention of de novo synthesized lipid. Carbohydrate, lipid, and protein disappearance across the estrous cycle, and following OVX are shown in Figure 4. In cycling animals, the average carbohydrate disappearance was increased in obese animals compared to lean (p=0.005). Lipid disappearance did not differ when averaged across the cycle (Figure 4b); however, it was significantly higher in the obese on the day of estrus (p=0.048, data not shown). No differences in protein disappearance were identified between lean and obese cycling rats. There were also no differences in substrate disappearance immediately following OVX, when animals were in energy balance (OVX-EB) (Figure 4c). In contrast, during the period of rapid, OVX-induced weight gain, obese animals had significantly higher protein disappearance than the lean (Figure 4d, p=0.025). Substrate disappearance was correlated with total activity during the period of OVX-induced weight gain. In lean animals activity levels were correlated with lipid disappearance (R=0.688, p<0.001); yet in the obese activity was correlated with carbohydrate disappearance (R=0.577, p=0.003).

**Plasma Measurements:** Levels of circulating glucose, free fatty acids (NEFA), cholesterol, triglycerides, and estradiol are shown in Table 1. Plasma glucose levels did not vary significantly between obese and lean animals; however, following OVX, glucose levels were elevated in both groups (p<0.001). Similarly, NEFAs did not vary significantly between lean and obese, but following OVX NEFA levels decreased in both groups (p=0.048). Triglycerides (TG) were the only factor that varied significantly between obese and lean animals, with obese
having higher TG than lean (p=0.008). While there was a slight rise in TG following OVX in both groups, this did not reach statistical significance. Neither obesity, nor OVX, altered the cholesterol levels. Finally, estradiol levels decreased significantly following OVX as expected following removal of the ovaries, but we saw no difference between lean and obese at the time points measured.

**DISCUSSION:**
This study is the first to present an in depth analysis of the effects of obesity on energy balance and fuel utilization in adult female rats, over the estrous cycle and during weight gain following surgical ovariectomy. In contrast to our original hypothesis, obesity was associated with a greater positive energy imbalance during D2 and P and a more substantial negative energy imbalance during E. The majority of these fluctuations in energy balance over the cycle in both lean and obese rats were driven by changes in energy intake rather than energy expenditure. Obesity, however, was associated with lower activity levels, yet NREE in the obese was similar to the lean, suggesting a higher energetic cost for each movement in the obese. Obese animals were also more dependent on carbohydrate over the cycle. Finally, OVX induced a large positive energy imbalance in both obese and lean rats, increasing energy intake in the lean to match that of the obese, while reducing expenditure in the obese to match that of the lean. These observations reveal an effect of obesity on energy balance, fuel utilization, and activity levels over the estrous cycle, and with loss of ovarian function, which may have implications for studies focused on obesity and energy balance in female rodents.

*EI over the cycle, the effect of obesity*
Our findings, that during estrus rats decreased their energy intake and increase activity, are consistent with the results of several other studies (4, 9, 17, 49). These cyclical changes in EI are generally thought to be driven primarily by changes in circulating estrogens (4). There are
no studies, to our knowledge, that have examined the impact of obesity on estrus-related changes in food intake. The data presented here demonstrate that the decrease in food intake commonly observed in response to rising estrogen levels is delayed in obese animals, compared to lean. We measured plasma estradiol in both groups in D2, when estradiol levels are beginning to rise in preparation for estrus. While we saw no difference between lean and obese at this time point, we cannot rule out an effect of obesity on circulating estradiol levels at other stages of the cycle. It is also possible that with the same hormonal profile, obese animals may be less sensitive to changes in estrogens over the cycle. Further studies with a more focused examination of estradiol’s regulation of energy balance will be needed to delineate the role of estrogens and estrogen sensitivity on obesity’s impact on food intake over the cycle.

**TEE over the cycle, the effect of obesity**

Activity levels have often been used as a surrogate for energy expenditure as measuring activity using running wheels is more logistically feasible for many research groups. The vast majority of studies demonstrate that running wheel activity increases during estrus (1, 9, 17), and we observed the same increase in spontaneous activity levels in the present study. The decreased activity we observed in the obese animals is also consistent with literature on the obesity-prone phenotype (40, 50). What is critical to highlight from the present observations, however, is that a lower activity level in the obese translated to a similar level of expended energy for lean and obese animals. In short, for a given change in activity, the energetic cost is greater in the obese than in the lean. These findings suggest that for obesity studies, lower activity levels in obese rats cannot simply be translated to lower levels of expended energy. However, this assertion needs to be confirmed in a system with a rapid response time and measures of true basal metabolism (in the fasted state) (8, 39).

Few studies have measured energy expenditure in rodents across the estrus cycle, and no
other studies have examined the impact of obesity on estrus-related changes in TEE, NREE, and REE. In a study of young, rapidly growing rats (starting at 5 weeks of age), it was shown that TEE did not change with cycle day; however, when animals were given access to running wheels the authors saw a decrease in TEE during metestrus (equivalent to diestrus in this study) (1). Our studies did not provide the opportunity for this type of volitional activity, but only monitored spontaneous activity in the home cage. It would be interesting to repeat the present study with a running wheel option, as a more substantial impact may be observed in NREE, REE, and TEE. In a separate study, REE (measured over 2 hours of the light cycle) was reportedly higher in E than in D in a cohort of young adult rats (less than 300 g) (43). We observed a similar phenomenon, but the impact of this change on overall energy balance was minimal. In general, TEE remained relatively stable over the cycle, and this appears to be due to the offset cycles of food intake (affecting the thermic effect of food) and physical activity (affecting activity thermogenesis).

It is important to note our animals were fed a high fat diet for most of their lives. At this level of dietary fat, we have observed that an acute bout of HF feeding increases TEE, NREE, and REE, in obesity-resistant, but not obesity-prone, rats (26), suggesting that a more pronounced effect over the cycle may have been masked by this long term dietary regimen. Moreover, the component analysis was performed in the ad libitum fed animal. As such, our estimate of REE is not a measurement of basal metabolism (REE measured in fasted animals); rather, it reflects the average level of expended energy during a time when the animal is at rest, and usually sleeping. This deviation from basal metabolic measurements becomes particularly apparent during OVX-induced weight gain when animals are overfeeding. During this time, the rats are likely to have a substantial amount of food in their digestive tract throughout the entire day, and REE will include some component of the thermic effect of food.
**Energy Balance over the cycle, effect of obesity**

When averaged across their cycle, female rodents in this study exhibited a minor positive energy imbalance. The timing of the fluctuation in EB across the estrous cycle suggests that it this is an estrogen mediated phenomenon, and there is substantial evidence to support this notion (15, 17). Whether the effect of obesity on energy balance is mediated through estrogens is less clear (22). While we did not see a difference in D2 estradiol levels between lean and obese, blood draws across the entire cycle may have revealed an effect of obesity. Estrogens do not appear to directly alter leptin levels (44); however, their impact on leptin signaling pathways may be influenced by the reduced leptin sensitivity that exists in obese rats (31). The signaling pathways of leptin and estrogens are known to overlap, both in peripheral tissues (51) and in the hypothalamus (22). It is likely that the impact of obesity on the fluctuation in energy balance across the cycle is the result of the interplay between estrogens, leptin, and the sensitivity of the hypothalamus (or other tissues) to these hormones.

**Fuel Utilization over the cycle, effect of obesity**

The primary determinant of RER is energy balance, which explains the higher RER in obese rats on D2 and P. However, during estrus the obese are in a much greater negative energy imbalance relative to the lean, yet the RER of the two groups is approximately the same. This suggests that the obese may have an impaired ability to oxidize fat when faced with a metabolic state that typically ramps up the mobilization and utilization of fat for energy (a negative energy imbalance). Obesity is known to be associated with an impaired ability to regulate fat during metabolic stress, a characteristic that has been referred to as metabolic inflexibility (21). This characteristic of obesity, when overlaid upon the changes in energy balance that occur over the estrous cycle, may contribute to their obese phenotype. Over time, more ingested fat will be retained in adipose, while carbohydrate will provide a greater portion of the substrate for energy production. This preferential use of carbohydrate for energy needs while storing fat is an
energetically efficient way of gaining weight (18, 48).

**Surgical Ovariectomy – Effects on EI, TEE, EB**

Our study is consistent with overwhelming evidence that OVX induces weight gain. In general, the relative contribution of changing intake or expenditure to create this energy imbalance may vary with species or model, but the impact on energy balance is the same. While some studies explain the weight gain on an OVX-induced suppression of energy expenditure (46), others report that most of the difference is due to increased food intake (54). As stated previously, the decline in expenditure from reduced activity levels may be masked by an increased thermic effect of food associated with the hyperphagia and/or an increased basal energy requirement from the gained weight. These opposing effects on the components of total energy expenditure are often overlooked in the interpretation of data in the post-OVX state, and may explain why the OVX-induced decline in activity is not always accompanied by a decrease in TEE.

In previous studies, we observed that the OVX-induced period of rapid weight gain lasts approximately 3 weeks and that feed efficiency was lower for obese rats (33). Eventually the obese rats gained a similar amount of weight as the lean, but during this transient period of time, it appeared that the energy imbalance and the rate of weight gain were blunted in obese rats. Obesity from forced overfeeding resulted in a similar delay in OVX induced weight gain (41), and we tended to see the same phenomenon in the present study. It is possible to speculate that OVX-induced leptin insensitivity could play a role in this delayed weight gain in the obese; however evidence for such a mechanism is equivocal (13-15). We would hypothesize that this delayed rate of weight gain reflects an impaired ability of obese animals to clear and store the excess energy (33). The higher levels of circulating nutrients may feedback through known nutrient sensing systems to attenuate their drive to overeat. Insulin resistance is associated with an impaired ability to adjust metabolism in response to metabolic challenges,
like fasting, exercise, and overfeeding (20, 21, 28, 29). After OVX, this 3-week period of chronic overfeeding undoubtedly represents a substantial metabolic challenge. The lack of overfeeding-induced suppression of NEFAs and higher TGs in obese would suggest this as a plausible explanation, but a more thorough examination of metabolism during this time with in vivo tracers is required to characterize the response of obese rats to the metabolic challenge of OVX.

PERSPECTIVES AND SIGNIFICANCE

While the estrous cycle adds a level of complexity in conducting studies in female rodents, there are many research questions that can be addressed only through studying females. The results of this study highlight the importance of being aware of the stage of the estrus cycle when designing preclinical studies in females, particularly where design or outcomes include energy balance, obesity, and physical activity. In the present study, we observed three additional aspects of the obese phenotype in females, which may play a relevant role in health and disease. First, despite lower activity levels, the obese essentially expended the same amount of energy as the lean. Second, obese rats exhibited a more dramatic fluctuation between the extremes of energy balance throughout the estrous cycle. Third, obese rats tend to have a blunted OVX-induced energy imbalance, which explains, in part, previous observations of lower feed efficiency and delayed weight gain after OVX (33, 41). We suspect that the relative contributions of EI or TEE to the changes over the cycle or in response to OVX may vary with diet, housing conditions, strain, species, or even between individuals, but that the overall impact of obesity on inducing positive energy imbalance is likely to prove more consistent.
ACKNOWLEDGMENTS

This research was supported by a University of Colorado Denver Thorkildsen fellowship to E.D.G., an American Institute for Cancer Research fellowship to E.D.G., and by NIH grant DK038088 to P.S.M. We also appreciate the assistance of the Colorado Nutrition and Obesity Research Center’s Energy Balance Laboratory and Metabolic Core (NIH DK48520).
REFERENCES:


34. MacLean PS, Higgins JA, Jackman MR, Johnson GC, Fleming-Elder BK, Wyatt HR, Melanson EL, and Hill JO. Peripheral metabolic responses to


FIGURE LEGENDS:

**Figure 1:** Energy balance, energy intake, and total energy expenditure. Data are shown for lean and obese rats during each phase of the estrus cycle (D1 – diestrus 1; D2 – diestrus 2; P – proestrus; E – estrus), immediately following OVX surgery while in energy balance (OVX-EB), and during the period of OVX induced rapid weight gain (OVX-Gain). (A) Energy Balance. (B) Energy Intake. (C) Total Energy Expenditure. The effects of obesity, cycle day, and their interaction were examined by ANOVA, with planned comparisons between lean and obese groups. Significant difference between Lean and Obese groups is indicated by *, p<0.05.

**Figure 2:** Component analysis of total energy expenditure. Total energy expenditure (TEE) was divided into its (A) Resting (REEp) and (B) Non-resting (NREEp) components. Data are shown for lean and obese rats during each phase of the estrus cycle (D1 – diestrus 1; D2 – diestrus 2; P – proestrus; E – estrus), immediately following OVX surgery while in energy balance (OVX-EB), and during the period of OVX induced rapid weight gain (OVX-Gain). The effects of obesity, cycle day, and their interaction were examined by ANOVA.

**Figure 3:** (A) Total Activity for lean and obese rats during each phase of the estrus cycle (D1 – diestrus 1; D2 – diestrus 2; P – proestrus; E – estrus), immediately following OVX surgery while in energy balance (OVX-EB), and during the period of OVX induced rapid weight gain (OVX-Gain). The effects of obesity, cycle day, and their interaction were examined by ANOVA. (B) Activity vs NREE for both obese (solid squares) and lean (open squares). NREE positively correlated with total activity in both the lean (R=0.413, p<0.001) and the obese (R=0.510, p<0.001), and the slope of the regression curve for this relationship was significantly greater in the obese than in the lean (slope 0.0006 vs 0.0001, p<0.001).
Figure 4: (A) Non-protein Respiratory Exchange Ratio (RER) for lean and obese rats during each phase of the estrus cycle (D1 – diestrus 1; D2 – diestrus 2; P – proestrus; E – estrus), immediately following OVX surgery while in energy balance (OVX-EB), and during the period of OVX induced rapid weight gain (OVX-Gain). (B) Carbohydrate, Lipid, and Protein disappearance for lean and obese animals, averaged across the cycle. (C) Carbohydrate, Lipid, and Protein disappearance for lean and obese animals immediately following OVX surgery while in energy balance (OVX-EB). (D) Carbohydrate, Lipid, and Protein disappearance for lean and obese animals during the period of OVX induced rapid weight gain. Significant difference between Lean and Obese groups is indicated by *, p<0.05.
TABLE LEGENDS:

Table 1: Morphometric and Plasma Characteristics

Data are expressed as means ± SEM; BW, body weight; FFM, fat free mass; FM, fat mass; NEFA, non-esterified fatty acids. Body composition was determined through a combination of dual-energy X-ray absorptiometry (DXA) and quantitative magnetic resonance imaging (qMRI). Plasma was collected on D2 of the cycle (cycling animals) or at the time of sacrifice (OVX animals), and cholesterol, triglycerides, NEFAs, glucose, and estradiol were measured using standard assays. For rate of weight gain, all animals were included in the analysis (n = 20-25 per group); for plasma and some morphometric measurements, a representative subset of animals was used (n = 10-14 per group). Data were examined by ANOVA, with significance set at p<0.05. aObesity effect; bOVX effect.
Table 1: Morphometric and Plasma Characteristics

<table>
<thead>
<tr>
<th>Variable</th>
<th>Lean Cycling</th>
<th>Lean OVX</th>
<th>Obese Cycling</th>
<th>Obese OVX</th>
<th>Obesity Effect</th>
<th>OVX Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weight Gain (g/day) b</td>
<td>0.51 ± 0.49</td>
<td>2.91 ± 0.35</td>
<td>0.44 ± 0.30</td>
<td>3.01 ± 0.41</td>
<td>p = 0.976</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>BW (g) a</td>
<td>308.8 ± 7.4</td>
<td>318.1 ± 6.1</td>
<td>380.3 ± 14.0</td>
<td>390.4 ± 9.3</td>
<td>p &lt; 0.001</td>
<td>p = 0.342</td>
</tr>
<tr>
<td>FFM (g) a</td>
<td>237.6 ± 5.1</td>
<td>246.7 ± 3.9</td>
<td>268.9 ± 9.2</td>
<td>275.7 ± 6.1</td>
<td>p &lt; 0.001</td>
<td>p = 0.240</td>
</tr>
<tr>
<td>FM (g) a</td>
<td>71.2 ± 5.0</td>
<td>71.5 ± 4.9</td>
<td>111.4 ± 6.0</td>
<td>114.8 ± 6.2</td>
<td>p &lt; 0.001</td>
<td>p = 0.753</td>
</tr>
<tr>
<td>%FM a</td>
<td>22.9 ± 1.3</td>
<td>22.3 ± 1.2</td>
<td>29.2 ± 0.9</td>
<td>29.2 ± 1.2</td>
<td>p &lt; 0.001</td>
<td>p = 0.796</td>
</tr>
<tr>
<td>Glucose (mM) b</td>
<td>9.84 ± 0.5</td>
<td>12.95 ± 0.8</td>
<td>11.05 ± 0.7</td>
<td>14.02 ± 0.7</td>
<td>p = 0.207</td>
<td>p &lt; 0.001</td>
</tr>
<tr>
<td>Cholesterol (mM)</td>
<td>2.06 ± 0.3</td>
<td>2.11 ± 0.4</td>
<td>1.88 ± 0.3</td>
<td>2.34 ± 0.3</td>
<td>p = 0.953</td>
<td>p = 0.459</td>
</tr>
<tr>
<td>Triglycerides (mM) a</td>
<td>0.93 ± 0.1</td>
<td>1.08 ± 0.2</td>
<td>1.53 ± 0.3</td>
<td>2.43 ± 0.6</td>
<td>p = 0.008</td>
<td>p = 0.140</td>
</tr>
<tr>
<td>NEFA (uM) b</td>
<td>714.0 ± 79.6</td>
<td>433.4 ± 58.0</td>
<td>650.8 ± 55.3</td>
<td>602.8 ± 66.8</td>
<td>p = 0.546</td>
<td>p = 0.048</td>
</tr>
<tr>
<td>Estradiol (pM) b</td>
<td>167.2 ± 18.3</td>
<td>52.7 ± 8.6</td>
<td>187.6 ± 19.3</td>
<td>49.5 ± 5.3</td>
<td>p = 0.886</td>
<td>p &lt; 0.001</td>
</tr>
</tbody>
</table>
Figure 1

A

**Energy Balance (kcal/day)**

- Lean
- Obese

- Cycle effect: p<0.001

B

**Energy Intake (kcal/day)**

- Lean
- Obese

- Cycle effect: p<0.001

C

**Total Energy Expenditure (kcal/day)**

- Lean
- Obese

- Obesity effect: p<0.001
Figure 2

A

Resting Energy Expenditure (kcal/day)

<table>
<thead>
<tr>
<th>D1</th>
<th>D2</th>
<th>P</th>
<th>E</th>
<th>OVX</th>
<th>EB</th>
<th>OVX Gain</th>
</tr>
</thead>
</table>

Obesity effect: p<0.001
Cycle effect: p<0.001

B

Non Resting Energy Expenditure (kcal/day)

<table>
<thead>
<tr>
<th>D1</th>
<th>D2</th>
<th>P</th>
<th>E</th>
<th>OVX</th>
<th>EB</th>
<th>OVX Gain</th>
</tr>
</thead>
</table>

Obesity effect: p<0.05
Cycle effect: p<0.01
Figure 3

A

![Bar chart showing total activity (counts x 10^3) for different conditions.](chartA.png)

- **Lean**: Obesity effect: p<0.001, cycle effect: p<0.001
- **Obese**: Obesity effect: p<0.001

B

![Scatter plot showing non-resting energy expenditure (kcal/day) vs. total activity (counts/day).](chartB.png)

- **Obese**: y = 0.0006x + 9.6456, R^2 = 0.2504
- **Lean**: y = 0.0003x + 12.383, R^2 = 0.1326