Homeostatic Regulation of Estrus Cycle of Young Female Mice on Western Diet

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Abstract

The etiology of reproductive disorders correlate with weight gain in patients, but the link between reproduction, diet, and weight has been difficult to translate in rodents. As rates of childhood obesity and reproductive disorders increase, the need to study the effects of weight and diet on adolescent females is key. Previous studies show female mice are resistant to high-fat diet (HFD)-induced weight gain, but the mechanisms are unclear. Literature also suggests that ovarian function is essential to resistance in weight gain, as an ovariectomy leads to a weight gaining phenotype similar to male mice on a HFD. However, reproductive changes that occur in adolescent mice on HFD have not been assessed. Here, we show that regulation of the estrus cycle via progesterone is critical to metabolic homeostasis in female mice on a HFD. Female mice were put on HF or control diet for twelve weeks starting at four weeks of age. Every four weeks, their estrus cycle was tracked and fasting glucose was measured. We found that after four weeks on HFD, there was no difference in weight between groups, but an increase in time spent in proestrus and estrus in mice on HFD and an increase in serum progesterone during proestrus. These results show that intact females modulate their estrus cycle in response to a HFD as a mechanism of homeostatic regulation of body weight, protecting them from metabolic abnormalities. Understanding the mechanisms behind this protection may yield therapeutic opportunities for treatment of reproductive disorders in adolescent female patients.

Keywords (6 maximum): high-fat diet, females, estrus cycle, metabolism, reproduction, adolescence
Introduction

Regulation of metabolism and reproduction are highly connected. Both processes involve whole body changes in neuron and hormone signaling to produce dynamic regulation of cell activity and whole-body physiology [1-3]. Dysregulation of metabolism is a prominent feature of many reproductive disorders such as endometriosis and polycystic ovarian syndrome (PCOS) [4-7]. This is especially true for the development of reproductive disorders in adolescence, as metabolic abnormalities are a common characteristic of PCOS in adolescence[8, 9]. Furthermore, diet and neuroendocrine mediators are important factors that contribute to the phenotype of PCOS in adolescents[9]. Although chronic obesity can increase symptom severity of reproductive disorders, there is a well-defined link between short term increases in caloric intake and fertility. In mammals that breed throughout the year, acute increases in energy stores are linked to increased pregnancy rates and fetal health improvements [10]; however, chronic increases in caloric consumption have a negative impact on reproduction [11-13].

One difficulty in understanding the interaction between metabolism and reproduction is the metabolic sex difference seen in rodents. In wild-type mice, males on a high-fat diet gain weight at a significantly faster rate than female mice on the same diet; moreover, females are obesity-resistant [14, 15]. Previous literature has shown that ovariectomized females gain weight similarly to males [16, 17]. Despite evidence that ovarian function is involved in the protection from metabolic disturbances in female mice, there is little evidence to explain why this is the case [18, 19]. Steroid hormones may be an important mediator of this phenotype. Changes in serum concentrations of sex hormones, particularly progesterone, are important in understanding how consumption of high-fat diet may influence cycling [20, 21]. Most studies on the effects of high-fat diet occur after weight gain has set in and therefore miss early therapeutic windows in the development of pathological changes.
Here we use young female mice to show the early development of altered estrus cycling in mice fed a diet composed of 45% fat. Starting at four weeks of age, females were given either a control diet or a 45% high-fat diet, and estrus cycling was monitored for twelve weeks on diet. As expected, there were no differences in weight gain or glucose metabolism. We show that estrus cycling is altered at four, but not eight, weeks on high-fat diet such that young female mice on high fat diet display a transient increase in time spent in proestrus and estrus. Importantly, these changes in estrus cycling occur in the early stages of high fat diet, well before the development of excess weight gain. Increases in time spent during proestrus are mirrored by increases in serum progesterone during this phase at four, but not eight, weeks on high-fat diet. These results show that female mice have a homeostatic regulation of reproductive cycling when on high-fat diet, which provides novel evidence to help explain why female mice are resistant to high-fat diet induced weight gain.

Methods (1131 words)

Animals

Young adult female C57BL/6J mice were used for experiments. Mice were weaned at three weeks of age and put onto diet (high-fat diet or control chow diet) starting at four weeks of age for up to twelve weeks. The onset of sexual maturity served as the beginning of the diet change and persisted for eight weeks wherein cycling and weight fluctuations were evaluated. The high-fat diet (TD.130784, Teklad Custom Diet, Envigo) was 44.6% kcal from fat, 40.7% kcal from carbohydrates, and 14.7% kcal from protein. The control chow diet (LabDiet ProLab RMH 1800) was 5.0% kcal from fat and 18.00% kcal from protein. Animals were group housed in polypropylene cages and maintained at a room temperature of 21 ± 2°C under a 12h light-dark cycle (lights on from 0600h to 1800h) with ad libitum access to water and chow. Breeders were purchased from Jackson Lab and
bred in-house, those offspring were used to establish our in-house breeding colony and animals used in the study. Animals were sacrificed for histology experiments every four weeks of diet, up to twelve weeks. For the high-fat diet group, there were 14 animals through week 4, 10 though week 8, and 4 through week 12; For the control group, there were 12 animals through week 4, 8 animals through week 8, and 4 animals through week 12. All procedures were in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and approved by the University of Texas at Dallas Institutional Animal Care and Use Committee.

Mice were weighed weekly starting the week of diet assignment between 1600h and 1800h for twelve weeks. Fasting glucose levels were tested once every four weeks as a negative control, as changes in glucose metabolism are not expected during this period. Mice were fasted for 14 hours overnight prior to testing at 0800h. Blood glucose levels were measured using the AlphaTRAK Veterinary Blood Glucose Monitoring Kit.

**Assessment of Estrus Cycles**

Estrus cycle phases (proestrus, estrus, metestrus, and diestrus) were assessed by collecting samples via vaginal lavage twice per day, once at 0900h and once at 1800h, as described previously [21-23]. Mice were sampled for nine days every four weeks for up to twelve weeks on diet. Vaginal lavage was performed by flushing the vagina with 20µL of saline (0.9% saline, pH 7.4) until cloudy. Samples were dry-fixed overnight on charged microscope slides and stained with 0.1% Toluidine Blue O (Sigma-Aldrich, Cat#89640-5G) diluted in double-distilled deionized water (ddH2O) for two minutes, then sequentially incubated in ddH2O, 100% 200 proof ethanol (Decon Laboratories, CAS#64-17-5, Cat#2701), and 100% ClearRite-3 (Thermo Scientific, Cat#6901TS) for one minute each. Slides were mounted with EMS DPX Mountant for Microscopy (Electron Microscopy Sciences, Cat#13512) and imaged via brightfield microscopy using the Olympus VS120 Virtual Slide Microscope at 5x and 20x magnification.
Estrus cycle phases were classified by the cytology described in Cora, Kooistra, and Travlos (2015). Briefly, proestrus (Figure 1a,b) was classified by the appearance of primarily nucleated epithelial cells and some anucleated epithelial cells with very little to no neutrophils present. Nucleated epithelial cells are rounded in shape, with a dark nucleus and deeply stained cytoplasm. Proestrus typically lasts from 14-24 hours. Estrus (Figure 1c,d) was classified as the predominance of anucleated epithelial cells, sometimes appearing in large clumps. Anucleated epithelial cells are large and irregularly shaped, with faint Toluidine Blue staining. These cells are easily distinguished by their lack of nuclei. Estrus typically lasts approximately 48 hours. In young mice, the first instance of proestrus and estrus (the first appearance of anucleated epithelial cells) can be used as a marker for the first ovulation following vaginal opening [24]. Metestrus (Figure 1e,f) was classified by the appearance of nucleated and anucleated epithelial cells as well as the presence of neutrophils. Neutrophils are very small, rounded cells and have the distinguishing feature of multilobed nuclei. Neutrophils are significantly smaller than either class of epithelial cell mentioned above. Metestrus typically lasts from 12-24 hours. Additionally, smears classified as metestrus contain the highest cell density of all the phases. Diestrus (Figure 1g,h) was classified by the predominance of neutrophils, with few to no nucleated and anucleated epithelial cells present and a low cell density. Diestrus is the longest phase of the cycle and typically lasts for 48-72 hours.

Smears were classified in a randomized order such that the experimenter was blinded to both time and diet. Values for Figure 3 were calculated by dividing the number of smears in each phase by the total number of samples for each mouse (i.e., two smears in proestrus / twenty smears taken = 10% time in proestrus). All estrus cycle tracking data can be found in Table 1.

Quantification of the cell populations in vaginal smears across the cycle was performed to aid in sample classification [25, 26]. Toluidine Blue O stained samples from mice (n=5 per phase) on both HF and control diet were imaged at 10x magnification. Each cell type was manually counted using...
ImageJ software within the entire imaged area. The mean ± SEM number of cells counted in smears across the cycle can be found in Table 2.

**Progesterone ELISA**

Serum was isolated from tail blood collected between 0900h and 1100h, immediately following vaginal smear collection for nine-day sampling periods every four weeks (Figure 2a). Blood was collected by snipping the tip of the tail and gently massaging the tail. Microvette EDTA-coated tail vein capsules (Sarstedt, Cat#16.444.100) were used to collect blood from the tip of the tail. Serum was isolated via centrifugation at 15000rpm at 4°C for 15 minutes. Samples were immediately stored at -80°C until time of assay. Quantification of serum progesterone concentrations from mice in proestrus, estrus, and diestrus were assessed via Progesterone ELISA for Mouse/Rat from IBL America (Cat#IB79183). This assay is a competitive immunoassay with standards ranging from 0-100ng/mL and sensitivity of 0.156ng/mL. Mean intra-assay and inter-assay CV values are 7.4% and 9.1%, respectively, with limited cross-reactivity to other steroid hormones. The assay was performed according to the manufacturer's instructions.

**Statistics**

Statistical analyses were performed using Prism 8.4 (GraphPad Software, La Jolla, CA, USA). Data are reported as mean ± SEM. Weight gain, fasting glucose, estrus cycle, and serum progesterone concentration data were analyzed using analyzed using repeated-measures mixed-effects analysis with factors of time and diet. The mixed-effects analysis was used because some mice were sacrificed at four and eight weeks on diet for follow-up experiments. Post hoc Sidak’s multiple comparison tests were performed where appropriate to compare differences between diet at each timepoint. Pearson r was used for correlations between weight and cycle length with two-tailed p-value. A p-value of less than 0.05 was considered statistically significant.
A graphical representation of the experimental timeline can be found in Figure 2a. All graphics were produced using BioRender.com.

Results

**Female C57BL/6J mice are resistant to high-fat diet induced weight gain.**

Four-week-old female mice were given either a high-fat diet or control diet for twelve weeks (Figure 2b). There was no difference in weights or fasting glucose at the start of diet treatment. Mice were weighed weekly between 1700h and 1800h, just prior to the start of the dark cycle. All mice gained weight over the twelve-week period (time: F=86.81, p=0.0382). There was a significant effect of diet (F=5.492, p=0.0277) as well as a significant interaction between time and diet (F=4.612, p<0.0001); however, post hoc analysis showed no difference between weights at any of the timepoints measured. Blood glucose levels were measured following a 14-hour overnight fast once every four weeks (Figure 2c). There was no significant effect of high-fat diet on fasting blood glucose levels.

**High-fat diet induced estrus cycle changes correlated with weight**

Weight gain in females has been linked to dysregulation of the reproductive axis. To test whether the changes in estrus cycle seen at week 4 were due to weight gain, we plotted a correlation of weight and cycle length, measured as days between consecutive proestrus smears (Figure 2d). There is a significant correlation between body weight and cycle length (r=0.3843, p=0.0435, two-tailed, \( R^2=0.1477 \)), indicating a direct relationship between weight and cyclicity in females. To test whether the changes in estrus were due to a total increase in cycle length, the time taken to complete one full cycle was compared every four weeks of diet treatment (Figure 2e). There was no significant difference in cycle length at any of the time points measured, indicating that the increase in ovulation at week 4 of high-fat diet is independent of total cycle length. There was no effect of diet on rates of cyclicity, measured by the percentage of mice that completed one full estrus cycle during each sampling period (Figure 2f).
High-fat diet causes an acute increase in time spent in proestrus and estrus.

To test whether estrus cycling plays a role in the lack of weight gain seen in females on high-fat diet, we gave females on high-fat or control diet at four weeks of age. Estrus cycles were tracked for nine days, twice per day, starting every four weeks of diet treatment. Four-week-old female mice on high-fat diet do not reach sexual maturity earlier than mice on a control diet, as there is no difference in the age at first ovulation measured by the first appearance of cornified epithelial cells, which occurred at approximately four and a half weeks of age in both groups (Table 1). It should be noted that at the start of the experiment, not all mice displayed vaginal opening, which typically occurs between 24 and 30 days of age [27, 28]. There were four out of 14 animals in the high-fat diet group and three out of 12 animals in the control group that did not display vaginal opening at the start of the experiment; however, all mice displayed vaginal opening by five weeks of age.

There were significant effects of both time (F=9.716, p=0.0006) and diet (F=5.800, p=0.0241), as well as a significant interaction between time and diet (F=5.989, p=0.0016) for time spent in proestrus (Figure 3a). There was a significant effect of time (F=10.11, p<0.0001), but no effect of diet or interaction between time and diet for time spent in estrus (Figure 3b). After consuming a high-fat diet for four weeks, there was a significant increase in the amount of time spent in proestrus (p=0.0012, compared to control) and in estrus for mice on high-fat diet (p=0.0287, compared to control). By week eight of diet, there was no difference in the time spent in proestrus or estrus between control and high-fat diet, indicating that high-fat diet initially causes an increase in ovulation that is normally seen at a later age. There were no significant differences in metestrus (Figure 3c) or diestrus (Figure 3d) at any of the time points measured; however, there was approximately a ten percent decrease in time spent in diestrus at four weeks on high fat diet compared to control. Although this decrease was not statistically significant (p=0.0793, HFD vs control at Week 4), as diestrus is a long-lasting phase typically extending over multiple days. The decrease in time spent in diestrus accompanies increases seen in proestrus and estrus phases, and...
accounts for the lack of changes seen in total cycle length, described above. At four weeks on diet, we see an increase in the total time spent in proestrus and estrus with a decrease in the time spent in diestrus. There were no significant differences in any phase at eight or twelve weeks on high-fat diet, indicating a compensatory role of estrus cycling in maintaining metabolic homeostasis in female mice.

Acute increase of serum progesterone during proestrus and estrus on high-fat diet.

Since we observed increases in the time spent in proestrus and estrus during week four of high-fat diet, we assessed serum progesterone levels across the cycle (proestrus, estrus, and diestrus) every four weeks on high-fat diet. The standard curve had a $R^2$ value of 0.9979. During proestrus (Figure 3e), there was a significant effect of diet on serum progesterone levels (diet: $F=86.60, p<0.0001$) and a significant interaction between time and diet on serum progesterone levels (time x diet: $F=36.07, p<0.0001$). There was an increase in serum progesterone at four weeks on diet, corresponding to the increased time in proestrus at this timepoint. During estrus (Figure 3f), there was a significant effect of diet on serum progesterone levels (diet: $F=8.292, p=0.0093$) and a significant interaction (time x diet: $F=6.905, p=0.0023$); however, we did not see differences in serum progesterone levels at four weeks on diet when we saw an increase in time spent in estrus. Interestingly, there was an increase in progesterone in mice on high-fat diet at the twelve-week timepoint, potentially pointing to long-term changes in progesterone during high-fat diet. During diestrus, there was a significant effect of diet on serum progesterone levels ($F=8.819, p=0.0076$); however, there were no significant post-hoc differences observed (data not shown).
Discussion

We show the novel finding that alterations in estrus cycling occurs in female C57BL/6J wild-type mice on high-fat diet at four weeks on a high-fat diet without changes in weight and glucose metabolism. Interestingly, we also show that estrus cycling returns to normal at eight weeks on diet. These findings bring to light a mechanism by which females regulate their metabolism. Our results for whole body metabolic measures, body weight and glucose, corroborate previously published findings that females are resistant to metabolic changes on high-fat diet [14-16]. We believe that the homeostatic regulation of estrus cycling in the early stages of high fat diet is an important and novel mechanism for female metabolic regulation [17].

Changes in estrus cycling occurred specifically within the proestrus and estrus phases, with no significant effect on metestrus and diestrus. Proestrus and estrus have a direct role in fertility and sexual maturity as acute hormonal changes and ovulation occur within these phases [29]. We show that females on high-fat diet initially have longer proestrus and estrus phases, which indicates increased fertility potential as these phases indicate sexual receptivity and ovulation [22, 29]. At week four, we also saw a decrease in the time spent in diestrus, though not statistically significant. Taken together, increased time spent in proestrus and estrus and decreased time spent in diestrus point towards increased fertility potential in mice on acute high fat diet. The disappearance of this enhanced proestrus phase by eight weeks on diet shows that females can adjust their reproductive cycling, similar to the adjustments seen in other cyclic behaviors such as feeding [13]. We also show that the changes in estrus cycle are moderately correlated to changes in weight and time on diet, such that heavier females have longer estrus cycles; however, the changes in estrus cycling occurred prior to any significant weight gain from diet.

There are several possible mechanisms for changes in estrus cycling prior to significant weight gain. Changes in serum progesterone are helpful in understanding how consumption of a diet high in fat may influence steroid hormones and cycling, which precedes reproductive disorders [9]. We found
that changes in serum progesterone during proestrus corresponds to observed changes in estrus
cycling for mice on high-fat diet, which indicates a role of progesterone in modulating cycling during
high-fat diet.

The strengths of this work primarily lie in the repeated assessment of estrous cycling in the same
mice over a twelve-week period. Changes seen in estrous cycling are therefore due to changes in the
cycling patterns of individual animals rather than inter-animal variability. We assessed cycle phases
twice per day, which allows us to capture shorter phases that may be missed when sampling only
once per day. We have also shown that changes in serum progesterone occur during the same time
as changes in estrus cycling, which strongly supports the changes observed in estrus cycling patterns.
Furthermore, all mice were bred in house which allows us to accurately determine the age of mice at
the start of the experiment and reduces potential variability due to ordering and shipping of mice
from outside laboratories. Finally, smears were classified by two different experimenters in a
randomized order, such that the experimenters were blinded to diet and the length of time the
mouse had been on diet.

A limitation of this work is that we are unable to assess additional hormones and other peripheral
metabolic markers. We chose arguably, one of the most reliable and important regulators of estrus
cycle and reproductive health (progesterone); however, assessment of additional hormones would
 elevate understanding of the interactions between diet and reproductive cycling prior to the onset
of obesity.

We believe these results provide an interesting and novel mechanism behind the common finding
that female mice are resistant to weight gain and other metabolic abnormalities on high-fat diet.

Our study provides a new look at female reproduction on western diet, and highlights the need for
female rodent models, adolescent and adult, in studies on high-fat diet and reproduction. Our study
also demonstrates the need to study phenotypes altered by high-fat diet prior to weight gain, as the
body undergoes changes in physiology prior to the onset of obesity.
Data Availability

The datasets generated during and/or analyzed during the current study are not publicly available but are available from the corresponding author on reasonable request.
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Figure 1. Representative vaginal smears from each phase of the estrus cycle. Representative vaginal smears from proestrus (a,b), estrus (c,d), metestrus (e,f), and diestrus (g,h) stained with Toluidine Blue O. Proestrus is characterized by nucleated epithelial cells (b, arrows). Estrus is characterized by cornified epithelial cells (d, arrows). Metestrus is characterized by high cell density and a combination of epithelial cells and neutrophils. Diestrus is characterized by a predominance of neutrophils (h, arrows). Original objective magnification of 5x (Panels a, c, e, g) or 20x (Panels b, d, f, h).

Figure 2. Female C57BL/6J Mice are Initially Resistant to High-Fat Diet-Induced Weight Gain. (a) Representation of the experimental timeline. (b) Body mass (mean g ±SEM) of female mice fed either a high-fat diet or control chow diet for 12 weeks. Diet was assigned at four weeks of age. All mice gained weight over the 12-week period (time: F=86.81, p=0.0382), with a significant interaction between time and diet (F=4.612, p<0.0001). (c) There was no significant difference in mean (± SEM) fasting glucose levels measured at every four weeks on high-fat diet. High-fat diet: blue, solid line; Control diet: red, dashed line; Weeks 0-4: n=14, Weeks 5-8: n=10, Weeks 9-12: n=4. Control diet: red, dashed line; Weeks 0-4: n=12, Weeks 4-8: n=8, Weeks 9-12: n=4. (d) There is a significant correlation (r = 0.3843, p = 0.0435, two-tailed) between weight and the time taken to complete one estrus cycle (proestrus to proestrus), but there is no significant interaction of time and diet on estrus cycle length (e). Values are represented as mean days (± SEM) to complete one estrus cycle during each sampling period. Acyclic mice were excluded from this analysis. There is no effect of diet rates of acyclicity (f). Values are the percentage of mice with at least one complete estrus cycle during each sampling period.

Figure 3. Short-term high-fat diet causes changes in estrus cycling patterns and serum progesterone levels. Representation of proportion of samples (mean number of samples ± SEM) spent in proestrus (a), estrus (b), metestrus (c), and diestrus (d) measured over nine days every four weeks on high-fat diet. For proestrus, there was a significant interaction between time and diet (F=5.989, p=0.0016) with Sidak’s post hoc multiple comparisons showing a significant difference in
proestrus at four weeks on diet (\(**p=0.0012\)). For estrus, there was a significant effect of time (\(F=10.11, p<0.0001\)), but no significant interaction (\(p=0.0915\)). Sidak's post hoc multiple comparisons showed a significant difference in time spent in estrus at four weeks on diet (\(*p=0.0287\)). No differences were seen in metestrus or diestrus. There was no significant difference between the groups at the start of diet treatment. Serum progesterone was measured using ELISA. (e) There is an increase in progesterone during proestrus at four weeks on high-fat diet but at no other timepoints. (f) There is an increase in progesterone during estrus at twelve weeks on high-fat diet but at no other timepoints. High-fat diet: blue; Control diet: red. n=3/diet for each timepoint. High-fat diet: Weeks 0-4: n=14, Weeks 5-8: n=10, Weeks 9-12: n=4. Control diet: red; Weeks 0-4: n=12, Weeks 4-8: n=8, Weeks 9-12: n=4. * indicates comparison to control diet. (g) Representative cycles from mice on HF and control diet at each timepoint. P=proestrus. E=estrus. M=metestrus. D=diestrus. Shading indicates samples collected during the dark cycle.
Table 1. Estrus cycle tracking of female mice on high fat diet for twelve weeks.

Footnotes:
P=proestrus; E=estrous; M=metestrus; D=diestrus. Black boxes indicate samples taken during the dark cycle. White boxes indicate samples taken during the light cycle. Samples from individual mice are organized into columns. *indicates first appearance of anucleated epithelial cells.

|          | High Fat Diet | Control |
|----------|---------------|---------|
| Week 0   |               |         |
| Day 1    | D M M D D D M | M D D D |
| Day 2    | D D D D D D M | D D D M |
| Day 3    | D D D M M D  | D D M D |
| Day 4    | D D E D D E  | M E M D |
| Day 5    | D E D E D E  | M M M D |
| Day 6    | D D D D D M  | D D M D |
| Day 7    | D D D D D D  | D D E D |
| Day 8    | E E E E E E  | E E E M |
| Day 9    | D D D M M M  | D D D M |

|          | Week 12 |
|----------|---------|
| Day 1    | E M M E D | M P D D D |
| Day 2    | E D D D E | M M D D D |
| Day 3    | D D D D D | D E D D D |
| Day 4    | D D D D E | D E D D D |
| Day 5    | D D D D D | D E D D D |
| Day 6    | D D D D D | D E D D D |
| Day 7    | E E E E E | E E E E E |
| Day 8    | D D D M D | D D D M M |
| Day 9    | D D D M D | D D D M M |

*Week 0 Week 12
Table 2. Quantification of cell populations across the estrus cycle.

Footnotes:
Mean (± SEM) number of cells in Toluidine Blue O stained vaginal smears. n=5 per phase. Samples were quantified from mice of both diets.

| Phase  | Nucleated Epithelial Cells | Anucleated Epithelial Cells | Neutrophils     |
|--------|----------------------------|------------------------------|-----------------|
| Proestrus | 8.6 ± 3.9                 | 7.3 ± 3.3                    | 71.6 ± 29.5     |
| Estrus  | 1.9 ± 0.8                  | 118.9 ± 10.5                 | 46.3 ± 10.5     |
| Metestrus | 6.6 ± 3.5                  | 24.1 ± 9.4                   | 326.0 ± 157.9   |
| Diestrus | 1.9 ± 1.1                  | 5.3 ± 1.8                    | 129.1 ± 24.4    |
Figure 1.
Figure 2.
Figure 3.

(a) Proestrus

(b) Estrus

(c) Metestrus

(d) Diestrus

(e) Proestrus

(f) Estrus

(g) Table 3

| Time on Diet (Weeks) | 0  | 4  | 8  | 12 |
|----------------------|----|----|----|----|
| Day 0               | D  | D  | D  | D  |
| Day 4               | P  | E  | E  | D  |
| Day 8               | M  | M  | M  | M  |
| Day 12              | D  | D  | D  | D  |

Notes: D = Control, P = High Fat Diet.