The Estrous Cycle of the Ewe Is Resistant to Disruption by Repeated, Acute Psychosocial Stress

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Short title: Estrous cycle resistance to psychosocial stress
Summary sentence: Repeated exposure to acute psychosocial stress does not interfere with the preovulatory LH surge or other estrous cycle parameters in the ewe.
Key words: Estrous cycle, follicular phase, LH surge, stress, cortisol, food restriction

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Abstract
Five experiments were conducted to test the hypothesis that psychosocial stress interferes with the estrous cycle of sheep. First, ewes were repeatedly isolated during the follicular phase. Timing, amplitude and duration of the preovulatory LH surge were not affected. In experiment 2, follicular phase ewes were subjected twice to a “layered stress” paradigm consisting of sequential hourly application of isolation, restraint, blindfold, and predator cues. This reduced LH pulse amplitude but did not affect the LH surge. In experiment 3, different acute stressors were given sequentially within the follicular phase: food denial plus unfamiliar noises and forced exercise, layered stress, exercise around midnight, and transportation. This, too, did not affect the LH surge. In experiment 4, variable acute psychosocial stress was given every 1-2 days for two entire estrous cycles; this did not disrupt any parameter of the cycle monitored. Lastly, experiment 5 examined whether the psychosocial stress paradigms of experiment 4 would disrupt the cycle and estrous behavior if sheep were metabolically stressed by chronic food restriction. Thirty percent of the food restricted ewes exhibited deterioration of estrous cycle parameters followed by cessation of cycles and failure to express estrous behavior. However, disruption was not more evident in ewes that also encountered psychosocial stress. Collectively, these findings indicate the estrous cycle of sheep is remarkably resistant to disruption by acute bouts of psychosocial stress applied intermittently during either a single follicular phase or repeatedly over two estrous cycles.

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Introduction
Disruptive effects of stress to gonadotropin secretion and reproductive function are well established [1-5]. Severe perturbations to homeostasis, such as immune/inflammatory stress, interfere with the ovulatory cycle in species ranging from rodents to ruminants to primates [6-9]. Less invasive disturbances, such as psychosocial stress, suppress gonadotropin secretion but there is little definitive evidence that, by themselves, they interfere with the ovarian cycle. Given the pervasive nature of psychosocial stress in today’s society, it is important to understand the extent to which this type of stress impacts reproductive fitness as well as the underlying mechanisms. Functional hypothalamic amenorrhea, a common menstrual cycle disorder in humans, has been attributed to psychosocial stress [10-12], but direct evidence that this reflects a stress response is lacking. More definitive evidence in monkeys indicates extended psychosocial stress combined with other stressors can interfere with reproductive hormone secretion and disrupt the menstrual cycle [13,14]. In sheep, the preovulatory LH surge can be delayed by truck transport [15], but there is no evidence that this type of psychosocial stress disrupts the estrous cycle.

We have determined that psychosocial stress inhibits pulsatile LH secretion in ovariectomized sheep by reducing both GnRH pulse amplitude [16] and pituitary responsiveness to GnRH [17]. Although the suppression of pulsatile GnRH secretion is independent of cortisol action [16], inhibition of pituitary responsiveness is caused by the concurrent rise in plasma cortisol [17]. To gain a better understanding of the impact of psychosocial stress on reproductive function and the mediatary role of cortisol and other factors in this response, we aimed to develop a model whereby psychosocial stress interferes with the estrous cycle of sheep. To this end, we conducted a series of experiments to test effects of short-term and prolonged exposure to acute bouts of various psychosocial stressors previously found to stimulate glucocorticoid secretion in sheep. This report describes our finding that the estrous cycle of the ewe is surprisingly resistant to disruption by psychosocial stress.

Materials and Methods
Five experiments were conducted in the breeding seasons of 5 years (Sep-Jan, 2002-2007) on mature Suffolk ewes maintained under standard husbandry conditions at the Sheep Research Facility near Ann Arbor, MI. The ewes were obtained from multiple suppliers and thus were of diverse genetic stock. Ewes were fed hay and alfalfa pellets and had free access to water and mineral licks. All expressed estrous cycles before use and none of the stressed ewes were previously exposed to a known stress. To enable groups of ewes to be studied simultaneously, the follicular phase of the cycle was synchronized using two intravaginal progesterone-releasing devices (CIDRs, Controlled Intravaginal Drug Release Devices; DEC International, Hamilton, NZ) as described elsewhere [18]. CIDRs maintain a luteal phase plasma progesterone concentration (2-4 ng/ml) and remain in place for 14 days (duration of the luteal phase). Upon CIDR removal, the follicular phase is initiated and the preovulatory LH surge occurs ~48 h later. Except as noted, ewes were not penned with rams during experiments and blood was sampled via indwelling jugular cannulae. Procedures were approved by the Committee for the Use and Care of Animals at the University of Michigan.

Psychosocial Stress Models
Nine psychosocial stress paradigms were applied variously across experiments. Pilot studies
indicated each of them enhanced cortisol secretion.

1. **Isolation**: Animals were individually moved from group housing to separate rooms to eliminate visual contact with other sheep (auditory cues still possible).

2. **Layered stress**: Ewes were exposed hourly to four cumulative stressors: isolation, restraint by confinement in a pen (0.5 x 1.2 m), blindfold to remove visual cues, predator threat by the sound of a barking dog played on a compact disc (CD).

3. **Food denial**: Animals were penned adjacent to flock mates being fed at the normal time. Experimental ewes were not fed until after completion of the stress (2 h).

4. **Noise/Exercise**: Animals were forced to walk around a room (5 x 6 m) for 15-20 min, followed by 5-10 min rest during which time random unfamiliar noises were played via CD (e.g., helicopter, jackhammer, exotic animal noises). This was repeated for 1-2 h.

5. **Circadian stress**: Ewes were awakened around midnight and forced to walk around a room (5 x 6 m) for 15-20 min, followed by 5-10 min of rest. This was repeated for 1-2 h.

6. **Transport**: Animals were transported in a livestock trailer on a variety of road surfaces and speeds for ~1.5 h. After a 30-45 min rest period, transport resumed for 1 h.

7. **Mock shear**: Ewes were restrained upright on the rump and electric shears were lightly moved around the body removing small amounts of wool from the midsection, head, fore and hind legs for 5 min. After a 1 h rest period, the process was repeated.

8. **Barking dog**: Two live dogs were brought into the room where ewes were penned to prevent physical contact. The dogs barked intermittently for 5 min and were then removed. The stress was repeated 45 min later.

9. **Blindfold/Barking dog CD**: Ewes were blindfolded and a CD of a barking dog was played for 5 min, repeated every 20 min, for 1-2 h.

**Experimental Designs**

Five experiments were performed to test if psychosocial stress disrupts the cycle. Because the outcome of each experiment dictated the design of subsequent ones, the rationale, purpose and design of each experiment are presented in *Results*.

**Assays**

LH was measured in duplicate aliquots of plasma (5-200 μl) using a modification [19] of a previously described RIA [20,21] and is expressed in terms of NIH-LH-S12. Intra- and inter-assay coefficients of variation were 5.0 and 6.6%, respectively, and assay sensitivity averaged 0.6 ng/ml (37 assays). Plasma cortisol was determined in duplicate aliquots (50 μl) using the Coat-a-Count cortisol kit (Siemans Healthcare Diagnostics Inc, Los Angeles, CA) validated for use in sheep [22]. Intra- and inter-assay coefficients of variation were 7.3 and 8.0%, respectively. Assay sensitivity averaged 0.7 ng/ml (32 assays). Progesterone was measured in duplicate 100-μl aliquots of plasma using the Coat-a-Count progesterone kit (Siemens Healthcare Diagnostics Inc, Los Angeles, CA) previously validated for use in sheep [23]. Intra- and inter-assay coefficients of variation were 8.2 and 9.3%, respectively, and assay sensitivity averaged 0.2 ng/ml (62 assays). Glucose was assayed in duplicate 10-μl plasma aliquots by a Glucose Oxidase based colorimetric assay (Modified Trinder; Pointe Scientific Inc, Canton MI) validated for use in sheep [24].

**Data Analysis**

The LH surge was defined as a rise in plasma LH concentration exceeding 2 standard deviations
above the pre-surge baseline and maintained for at least 4 h. Latent period to the surge was the interval from progesterone withdrawal (CIDR removal) to LH surge peak; the peak was defined as surge amplitude. LH surge duration was defined as the interval from the surge onset (i.e., when the LH value exceeded 2 standard deviations of the pre-surge baseline) to the time that LH fell to 10% of the surge peak. Hormone concentrations were log-transformed prior to analysis to normalize variation across a broad range of values. LH pulses were identified by the Cluster pulse-detection algorithm [25]. As in our previous studies [26], peak and nadir cluster sizes were set at 1 and 2, and the t-statistic used to determine significant increases or decreases in hormone concentration was 2.6. LH pulse amplitude was defined as the difference between the peak and preceding nadir. Frequency was calculated as number of pulses per sampling period. Further details of statistical analyses are presented in **Results**. Significance was defined as $P < 0.05$.

### Results

#### Experiment 1: Does repeated isolation disrupt the preovulatory LH surge?

The follicular phase of the cycle was synchronized in two groups of ewes: non-stress control (n=6) and repeated isolation stress (n=6). Controls were housed separately as a group to avoid exposure to the stress group. Stressed ewes were housed together prior to CIDR removal (0 h) and individually moved to isolation rooms for 6 h on three occasions during a single follicular phase: 12-18 h, 24-30 h, and 36-42 h after CIDR removal. These times represent the early- and mid-follicular phase and immediate pre-surge period, respectively (LH surge peak expected ~48 h after CIDR removal). After each isolation, ewes were returned to their common room. Blood was sampled from both groups every 30 min from 2 h before to 2 h after each isolation to assay cortisol, and every 2 h thereafter until 84 h to monitor the LH surge. Prior work indicates isolation inhibits pulsatile LH secretion in ovariectomized ewes [FJ Karsch, unpublished] but effects on the preovulatory LH surge have not been described.

In control ewes, plasma cortisol remained low throughout sampling (mean ± SEM, 11.0 ± 1.8 ng/ml). In stressed ewes, cortisol increased from 13.5 ± 2.2 ng/ml before stress to 35.3 ± 4.1, 29.7 ± 4.0, and 35.0 ± 5.9 ng/ml during the three bouts of isolation, respectively ($P < 0.02$; pre-stress vs. stress as determined by repeated measures ANOVA). Peak values during stress (37.5 ± 1.0 ng/ml) averaged ~3-fold over basal levels. All ewes exhibited the preovulatory LH surge. Timing, amplitude, and duration of the surge did not differ between control and stress ewes as determined by Student’s t-test (Table 1A, Experiment 1).

#### Experiment 2: Does the layered stress paradigm disrupt the preovulatory LH surge?

Since experiment 1 revealed repeated exposure to the same stressor did not affect the incidence, timing, amplitude or duration of the preovulatory LH surge, we hypothesized that variable psychosocial stress would be disruptive. Thus, we applied the layered stress paradigm in which novel psychosocial stressors are sequentially added over a 4-h period (see **Methods**). This paradigm stimulates cortisol and inhibits GnRH and LH pulse amplitude in ovariectomized ewes [16,17]. In addition to monitoring cortisol and the LH surge, LH pulses were examined prior to the surge.

The follicular phase of the cycle was synchronized in two groups of ewes: non-stress control (n=4) and stress (n=4). Starting 12 h after CIDR removal, jugular blood for LH pulse analysis was sampled at 6-min intervals for 14 h; cortisol was assayed at 30-min intervals. Ewes in both groups were maintained under calm conditions for the first 4 h of sampling. At that point (i.e.,
16 h after CIDR removal) the stress group was exposed to the 4-h layered stress paradigm; controls remained in calm conditions throughout. Stressed ewes were then returned to a common room for 2 h and subjected to a second 4-h bout of layered stress (22-26 h after CIDR removal, 6-min sampling continued). Blood was then sampled every 3 h until 72 h to assess the LH surge.

Representative LH and cortisol profiles are shown in Figure 1. Cortisol remained at basal levels throughout sampling in non-stressed controls (mean ± SEM, 10.2 ± 2.0 ng/ml). Repeated measures ANOVA revealed mean plasma cortisol concentrations increased during the first exposure to the layered stress (8.7 ± 1.8 vs. 35.9 ± 4.7 ng/ml; pre-stress vs. stress, respectively; treatment x time interaction, P < 0.05). During the second stress exposure, mean cortisol tended to increase; values rose to only 17.6 ± 3.6 ng/ml (P = 0.08, compared to pre-stress values). Peak cortisol values during the second bout of stress were 43% less than peak values during the first exposure (28.0 ± 5.6 vs. 49.3 ± 8.6 ng/ml, respectively; P < 0.02 by paired t-test).

In controls, LH pulse frequency and amplitude did not change significantly during the frequent sampling period (Fig. 1; Table 2). In stressed ewes, mean LH pulse amplitude decreased by 50% during first stress period compared to pre-stress values (P < 0.05, repeated measures ANOVA). The second bout of stress, however, did not alter amplitude of LH pulses compared to pre-stress values (P > 0.05). No change in LH pulse frequency was detected in either group. Importantly, all ewes expressed the LH surge and there was no difference in surge timing, amplitude or duration as determined by Student’s t-test (Table 1B, Experiment 2).

Experiment 3: Does repeated, variable psychosocial stress disrupt the LH surge?

In experiments 1 and 2, neither repeated isolation nor the layered stress affected timing, amplitude or duration of the preovulatory LH surge. However, either the same stressor was repeatedly used (experiment 1), which might have caused habituation, or the stress encompassed only a limited (possibly insufficient) portion of the follicular phase (experiment 2). We thus hypothesized that repeated variable stress during a major portion of the follicular phase would interfere with the LH surge.

The follicular phase was synchronized in two groups: stress (n=10) and non-stress control (n=4). Stress ewes were exposed to five different stressors between 15 and 42 h after CIDR removal (0 h) as follows: 1) food denial combined with noise/exercise (15-18 h); 2) layered stress (22-26 h); 3) circadian stress (31-32 h); 4) transport (38-41 h); 5) mock shear (41-42 h). Blood was sampled every 20 min during stress to monitor cortisol and every 3 h from 26-72 h to assess the LH surge. Previous work suggests transport by itself, at the time used here, delays the preovulatory LH surge [15].

Each stress, except food denial, significantly increased mean plasma cortisol levels compared to immediate pre-stress values (paired t-test, Table 3). Although cortisol declined toward the initial pre-stress level between stresses, values immediately prior to the next stress generally remained elevated; e.g., values prior to the last stress were 2-fold greater than the initial pre-stress value (14.5 ± 2.2 vs. 7.2 ± 1.4 ng/ml, respectively; P < 0.05 by paired t-test). Despite the sustained cortisol response, sequential exposure to the five different stressors did not influence the occurrence, timing, amplitude, or duration of the LH surge (Table 1C, Experiment 3).
**Experiment 4: Does extended psychosocial stress disrupt the estrous cycle?**

Results to this point suggest repeated exposure to acute psychosocial stress during a single follicular phase does not disrupt the preovulatory LH surge, although such stress did increase glucocorticoid secretion and, as shown for the layered stress in experiment 2, inhibit pulsatile LH secretion. Work in monkeys indicates more prolonged psychosocial stress combined with other stressors could interfere with menstrual cycles [13,14]. Here we tested if prolonged exposure to acute bouts of psychosocial stress over the course of more than two cycles disrupts the estrous cycle of ewes. To reduce effects of habituation (i.e., desensitization during prolonged exposure to constant stress), the stressors were applied in random order.

Estrous cycles of 12 ewes were synchronized using CIDRs during the early breeding season (Sep). Ewes were randomly allocated to two groups: non-stress control (n=6) and stress (n=6). Daily blood samples were taken via jugular venipuncture to monitor progesterone for 5 consecutive estrous cycles (Oct-Dec). Both groups were kept under non-stress conditions for Cycles 1 and 2 to establish basal estrous cycle parameters. Beginning in the follicular phase of Cycle 3, the stress group was acutely subjected to six different stressors in random sequence every 1-2 days for 39 days (exceeding 2 cycles): barking dog, circadian stress, layered stress, mock shear, transport, noise/exercise. Between acute stress sessions, ewes were isolated as much as facilities and space would allow (~50% of the time). Non-stress controls remained in calm conditions. Blood for cortisol assay was sampled prior to and immediately after each acute stress. During the follicular phase of Cycle 4 (second stress cycle), samples were taken at 4-h intervals to assess pre-surge and surge LH secretion (4-h sampling in each ewe began when progesterone fell to at least 50% of the peak value as determined by daily progesterone assays). Thereafter, daily sampling continued under non-stress conditions in Cycle 5 to assess carryover effects, as observations in monkeys suggest stress effects may manifest in the subsequent cycle [8,13].

Nine estrous cycle parameters were examined. 1) Length of follicular phase/periovulatory period: days plasma progesterone concentration was < 0.5 ng/ml. 2) Luteal phase length: days plasma progesterone was ≥ 0.5 ng/ml. 3) Cycle length: sum of parameters 1 + 2. 4) Integrated luteal phase progesterone: sum of all values within a given luteal phase. 5) Peak luteal phase progesterone: average of three highest contiguous values within a given luteal phase. 6) Pre-surge LH value (Cycle 4): mean plasma LH values from the time progesterone fell to half maximal to onset of the LH surge. 7) Latency to LH peak (Cycle 4): time from half-maximal progesterone to apex of surge. 8) LH surge amplitude: maximal value during surge. 9) LH surge duration.

Plasma cortisol (weekly samples) remained low throughout the experiment in non-stress controls (mean ± SEM, 9.4 ± 0.4 ng/ml) and in stressed ewes during non-stress cycles (8.8 ± 0.9 ng/ml; Cycles 1, 2 and 5). Initially, cortisol was increased by acute stresses, but responses became progressively dampened until no elevation in cortisol was detected. In this regard, cortisol increased significantly following 8 of 14 stress occasions during the first 3 weeks of stress but on only 1 of 12 subsequent stress occasions (Fig. 2A). It should be noted, however, that peak cortisol responses were likely missed as the cortisol rise was monitored immediately after each stress session.
Overall, the mean plasma progesterone profile was similar in stressed and control ewes (Fig. 3A). Repeated measures ANOVA revealed no effect of stress on any estrous cycle parameter examined (no treatment effects or treatment x time interactions). Length of the cycle and its stages, as well as progesterone values, were consistent within groups and similar between groups across the 5 estrous cycles (Table 4). Further, pre-surge LH concentrations and LH surge parameters (Cycle 4) were not altered by stress (Table 5A, Experiment 4). Of note, in the first stress cycle, one ewe exhibited a prolonged luteal phase compared to the rest of the group (26 vs. 12.4 ± 0.5 d mean for other ewes); progesterone values for this ewe were not included in Figure 3A.

**Experiment 5: Does extended psychosocial stress disrupt the estrous cycle and sexual behavior of nutritionally restricted ewes?**

Experiment 4 did not reveal any deleterious effects of stress on the estrous cycle, with the possible exception of a lengthened luteal phase in one ewe during the first stress cycle, as mentioned above. This ewe was leaner and appeared to be in poorer body condition than the others. While conducting experiment 4, we became aware of a study in monkeys indicating that psychosocial stress alone had no effect on the menstrual cycle but was disruptive when combined with the metabolic stress of diet + exercise [14]. Collectively, these observations led to the hypothesis tested here: repeated, acute variable psychosocial stress would disrupt the estrous cycle in nutritionally compromised ewes.

Beginning 5 months prior to monitoring reproductive endpoints, 16 ewes were fed a restricted calorie diet designed to reduce body weight by 25% when estrous cycles began at onset of the breeding season (Sep). Daily caloric intake was initially lowered by reducing the standard diet by 25%, after which food restriction was individually adjusted to attain and maintain the targeted weight loss. Ewes were weighed weekly prior to daily feeding and monitored daily for adverse health effects (e.g., lethargy, emaciation, lack of social interaction). At the start of the breeding season, food restricted ewes were allocated to two groups balanced for body weight: diet only (n=8) and diet + psychosocial stress (n=8). Control ewes (n=8) were normally fed and non-stressed.

This experiment was conducted in the same manner as experiment 4 except carryover effects of stress were not monitored in Cycle 5, since experiment 4 indicated there were no such effects. Instead, reproductive behavior was monitored in Cycle 5 because recent evidence indicates psychosocial stress interferes with certain aspects of sexual behavior (proceptivity, attractiveness of ewe to ram) in ovariectomized ewes treated with hormones to induce estrus [27]. Also, blood was sampled at 3-h intervals in the follicular phases of both Cycle 2 (pre-stress) and Cycle 4 (stress) to obtain better resolution of pre-surge and surge LH secretion than in the previous experiment and to allow comparison within individuals before and during stress. Additional samples were collected prior to feeding at the start of Cycle 1 and the end of Cycles 3 and 5 to assay glucose.

To facilitate monitoring of reproductive behavior, CIDRs were inserted at the onset of the luteal phase in Cycle 5. Beginning 20 h after CIDR removal, ewes in the diet + stress group were individually moved from isolation rooms every 6 h to a pen containing a vasectomized ram and observed for the onset of estrus, defined as the first immobilization that allowed the ram to mount. Control ewes and ewes in the diet only group were walked from an adjacent pen into the
pen containing the ram for behavioral testing. After 3 min of observation, ewes were removed from the test area to avoid further interaction with the ram. Ewes in the diet + stress group were isolated between observations. Once ewes expressed receptive behavior, they were removed from the study. Observations continued until 68 h after CIDR removal, 6 h after onset of receptivity in the last control ewe.

**Metabolic indicators.** Ewes in the restricted diet group lost 22% of their initial mean (± SEM) body weight (80.0 ± 4.8 vs. 62.3 ± 3.5 kg). Diet + stress ewes lost 24% of their initial body weight (76.9 ± 4.5 vs. 57.7 ± 2.2 kg). Weight loss ranged from 13-32%. Weights of normally fed controls did not change (69.2 ± 2.4 vs. 70.6 ± 1.7 kg, start vs. end). Blood glucose levels were within the normal pre-feeding range in sheep [28] for all groups at each time monitored (~50 mg/dl).

**Cortisol.** Plasma cortisol (weekly samples) in control and diet only ewes remained low and unchanged throughout the experiment (7.1 ± 1.0 and 9.6 ± 1.2 ng/ml, respectively; P > 0.1 by repeated measures ANOVA). In diet + stress ewes, cortisol was also low during non-stress cycles (9.4 ± 0.6 ng/ml, Cycles 1 and 2). During stress (Cycles 3 and 4), cortisol increased following 5 of 34 acute stress sessions (paired t-test of pre vs. immediate post stress values; Fig. 2B), however, as in experiment 4, maximal cortisol responses were likely missed because samples were not taken during the actual stress periods. Unlike experiment 4, significant cortisol responses were not seen primarily in the initial stress periods.

**Cycle parameters.** All ewes began to express estrous cycles at the start of the breeding season. Mean daily progesterone profiles were similar between controls and experimental ewes that continued to express estrous cycles (Fig. 3B). In these ewes, repeated measures ANOVA revealed no group differences for any estrous cycle parameter monitored (Table 6) including pre-surge LH values and LH surge parameters (Table 5B, Experiment 5). Notably, 5 of the 16 nutritionally compromised ewes either became anovulatory as judged by lack of a luteal phase rise in progesterone or had severely disrupted cycles as judged by a follicular phase/periovulatory period duration exceeding twice the upper 95% confidence interval of control ewes (Fig. 4). Three of these ewes were in the diet only group and two were in the diet + stress group. One of these ewes (#12, diet only group) became anovulatory after Cycle 1 and was not monitored beyond the first missed cycle. Prior to cycles becoming severely disrupted or ceasing altogether in these ewes, cycle characteristics began to deteriorate as judged by a 32% decrease in both the peak and integrated plasma progesterone values in the luteal phase, a shortening of the luteal phase and a lengthening of the follicular phase (although total cycle length did not change) (Fig. 5). Average weight loss in animals that had disrupted cycles (18.6%) was no greater than that of the other nutritionally compromised ewes. No control ewes stopped expressing cycles during the course of the study.

**Sexual behavior.** There were no significant differences in time of estrus onset (first mount by the ram) among control, diet only and diet + stress groups as determined by repeated measures ANOVA (43.3 ± 3.4, 48.2 ± 6.1 and 54.0 ± 4.5 h after CIDR removal, respectively; mean ± SEM). However, when values in the diet only and diet + stress groups were combined (no significant difference between these groups), the latency to estrus was significantly increased compared to normally fed controls (51.4 ± 3.0 vs. 43.3 ± 3.4 h after CIDR removal, respectively;
P < 0.05 by Student’s t-test). Four ewes had not entered estrus (2 diet only, 2 diet + stress) by the end of the observation period (68 h) and were not included in the analysis. These were the same 4 ewes that either had severely disrupted estrous cycles or ceased to express cycles based on progesterone profiles (behavior was not monitored in the other ewe that stopped cycling, Ewe #12).

**Discussion**

In this study, we aimed to develop a model in which psychosocial stress disrupts the estrous cycle of sheep, a model that could be used to examine mechanisms and mediators of stress-induced suppression of reproductive activity. Neither repeated exposure to various acute psychosocial stressors during a single follicular phase (experiments 1-3) nor repeated exposure to such stressors over the course of two estrous cycles (experiments 4 and 5) altered the incidence, timing, amplitude or duration of the preovulatory LH surge. In all 5 experiments collectively, the overall mean latent period to the LH peak among 33 stressed ewes did not differ from that of 28 non-stressed controls (49 vs 47 h in stressed and control ewes, respectively). Similarly, the overall mean peak of the LH surge for all experiments was remarkably similar (166 and 174 ng/ml in stressed and control ewes, respectively). Thus, an effect of our stress paradigms on LH surge characteristics, if any, was negligible and not detectible by our sampling intervals (2-4 h across the 5 experiments).

The lack of an effect of psychosocial stress on the LH surge was unexpected based on earlier findings that one of the stressors employed, transport shortly before onset of the preovulatory LH surge, delayed the LH surge of the ewe [15]. Further, our stress paradigms elicited other neuroendocrine stress responses such as enhanced cortisol and reduced pulsatile LH secretion, both of which could negatively impact the cycle and the LH surge. For example, exogenous cortisol can disrupt the follicular phase of the cycle [29,30] and delay both the spontaneous [29,30] and estradiol-induced LH surge of the ewe [31]. These disruptive effects of cortisol, however, were observed with plasma cortisol increments considerably higher and more prolonged than those induced by stress in the present study. Therefore, the lack of effect of our psychosocial stress paradigms on LH surge characteristics, if any, was negligible and not detectible by our sampling intervals (2-4 h across the 5 experiments).

In addition to a lack of an effect on the LH surge, repeated exposure to various random psychosocial stressors over the course of two estrous cycles did not interfere with expression of the estrous cycle or the duration of its follicular and luteal phases, nor did it impair function of the corpus luteum as monitored by plasma progesterone concentrations (experiment 4). Although this suggests lack of an effect on mechanisms that underlie generation of the estrous cycle, it remains possible that the stress paradigms employed here could interfere with reproductive endpoints that were not monitored, such as ovulation rate, percentage of ewes that ovulate or of ovulated eggs that can be fertilized, implantation, etc. Further work would be needed to address this possibility.

Repeated exposure to variable, acute psychosocial stressors also failed to interfere with the estrous cycle when combined with the metabolic stress of caloric restriction (experiment 5). Although estrous cycles and sexual behavior were disrupted in 2 of 8 ewes receiving the combined stress, this cannot be attributed to psychosocial stress because the cycle and behavior
were similarly disrupted in 3 of the 8 metabolically stressed ewes not subjected to psychosocial
stress. Lack of an effect of psychosocial stress was unexpected based on studies in rhesus
monkeys in which chronic psychosocial stress combined with other stress types, including
metabolic stress, interfered with the menstrual cycle and reproductive hormone secretion [13,14].
Nevertheless, work in pigs suggests that repeated exposure to acute stress, as employed in the
current study, is insufficient to disrupt the cycle [32]. The possibility that duration of exposure
to stress might account for the differing results is considered below.

The cessation of estrous cycles in nearly one third of the food-restricted animals in experiment 5
(both groups combined) is noteworthy because ewes in our flock rarely stop expressing estrous
cycles once they commence at the onset of the breeding season. For example, cycles persisted
throughout the observation period spanning 5 estrous cycles in all 14 control ewes in
experiments 4 and 5 combined. Of interest, cessation of cycles was preceded by inadequate
corpus luteum function (reduced progesterone secretion and short luteal phase) and a prolonged
follicular phase. These observations are consistent with the stress-induced disturbances of
menstrual cycle characteristics reported in rhesus monkeys [13,14,33] and with the conclusion
that inadequate secretory function of the corpus luteum is an early stage of stress-induced
derangement of the ovulatory cycle [13,33]. In addition, the metabolically stressed ewes in the
present study exhibited a prolonged latent period to onset of sexual receptivity (both groups
combined).

Further work is needed to determine how metabolic stress interferes with corpus luteum function
and disrupts the estrous cycle and sexual behavior. Long-term caloric restriction of sheep was
previously reported to inhibit pulsatile LH secretion, which would be expected to negatively
impact the estrous cycle, but this was seen only with severe weight loss and deterioration of body
condition (e.g., 40% weight loss) [34,35]. Here, weight loss averaged considerably less (~25%)
and it was no greater in ewes that stopped cycling than in those that continued to cycle.
Although we did not characterize LH pulses in food-restricted ewes, the pre-surge plasma LH
concentration assessed by 3-hourly sampling, which reflects LH release during a time when LH
is primarily pulsatile, was not significantly lowered by reduced caloric intake. It would be useful
to determine if the food restriction paradigm used here alters LH pulse frequency and/or
amplitude as well as other aspects of estrous cycle regulation, e.g., FSH secretion, follicular
development and estradiol secretion, or responsiveness of behavioral centers or the LH surge
generating mechanism to the stimulatory effects of estradiol.

Our present findings raise an important question. How can we account for the finding that
metabolic stress interfered with the estrous cycle in some ewes whereas our psychosocial stress
paradigms were uniformly ineffective in this regard? We forward three explanations that alone
or in combination might explain this result: 1) desensitization to psychosocial stress over time
(i.e., habituation) [36,37]; 2) the nature or severity of the stressor; 3) duration of the stress.
Initial evidence for habituation was obtained in experiment 2 in which the layered stress
paradigm was applied twice during the follicular phase; significant stimulation of cortisol and
inhibition of LH pulses were seen only during first exposure to the stressor. Although we
attempted to minimize habituation in subsequent experiments by using differing psychosocial
stressors in random sequence, desensitization was still suggested by progressively declining
(experiment 4) or minimal (experiment 5) cortisol responses, but it should be emphasized that cortisol was not monitored at the expected time of peak responses in those experiments.

Based on the cortisol response, however, there was no sign of habituation in experiment 1, yet repeated isolation did not interfere with the LH surge. This points to our other explanations for the lack of cycle disruption: nature/severity and duration of the stress. Regarding the nature or severity of the stress, it is noteworthy that another stress type, acute immune/inflammatory stress modeled by a 26-h infusion of endotoxin, disrupted the follicular phase of sheep by inhibiting pulsatile LH and estradiol secretion and by delaying or preventing the LH surge and estrous behavior [7]. Endotoxin infusion also stimulated cortisol secretion for at least 26 h. That our psychosocial stress paradigms were not as severe is suggested by a smaller plasma cortisol elevation (<40ng/ml) compared to that observed with endotoxin (>100 ng/ml) [7], and the cortisol rise was not sustained through the stress period. Thus, the psychosocial stressors used here might have been relatively weak compared to endotoxin and insufficient to disrupt the estrous cycle.

The lack of an effect on the cycle might also have been due to the duration of the stress. In all experiments, we applied psychosocial stress acutely and intermittently. Although the stress period spanned two estrous cycles in experiments 4 and 5, the duration of any individual stress session during that period might not have been sufficient to elicit disruptive effects, and the intermittent nature of the stress might have allowed the ewes to recover from one bout of stress before the next one was applied. In contrast, the metabolic stress paradigm, which disrupted the cycle in some ewes, was continuous and initiated 5 months before reproductive endpoints were monitored, and it continued for several more months during the observation period. It may well be that the estrous cycle of the ewe would be disrupted only in response to chronic psychosocial stress.

Despite the potential importance of duration, it should be pointed out that we are not aware of any definitive evidence that psychosocial stress by itself interferes with the cycle. Even with the aforementioned studies in primates, in which chronic psychosocial stress was disruptive, its efficacy alone was either not tested [13] or psychosocial stress had to be combined with other stress types to disrupt the cycle [14]. Perhaps the drive to reproduce is so strong that compensatory mechanisms, such as habituation, diminish the ability of psychosocial stress to interfere with cyclicity. This might be especially true in seasonal breeders, such as sheep, in which the physiologic drive and selection pressure to take advantage of the window of opportunity to reproduce is so intense that perturbations caused by the type of stressors used here are insufficient to interfere with ovulation. Seasonal changes in both reproductive neuroendocrine and glucocorticoid responses to stress have been observed [38-41], but their relevance to estrous cycle disruption has not been investigated.

In conclusion, the estrous cycle of the ewe appears to be remarkably resistant to disruption by acute bouts of psychosocial stress, whether applied intermittently during a single follicular phase or repeatedly over the course of several estrous cycles. This contrasts to the disruptive influence of a more severe stress type, immune/inflammatory stress [7], or chronic metabolic stress caused by caloric restriction (experiment 5). Due to an increasing awareness of the consequences of psychosocial stress on a myriad of physiological processes, including reproduction, our findings
encourage further work to address why the psychosocial stress models employed here failed to interfere with cyclicity as well as to explore whether chronic psychological stress interferes with the estrous cycle in this species.

Acknowledgements
We thank Doug Doop and Gary McCalla for their expert animal care and Morton Brown, Alison Cooper, Jennifer Davis, Kari Dugger, Loren Heller, James Lee, Christopher McCrum, Sarah Musil, Andrew Pytiak, Harold Render, Dustin Robinson, Juho Won, and Britta Wunderlich for their assistance in the planning, conducting and interpreting of the experiments. The authors are also grateful to Drs. Gordon D. Niswender and Leo E. Richert, Jr. for supplying LH assay reagents.

References
13. Xiao E, Xia-Zhang L, Ferin M. Inadequate luteal function is the initial clinical cyclic defect in a 12-day stress model that includes a psychogenic component in the rhesus monkey. J Clin Endocrinol Metab 2002; 87(5):2232-2237.

Figure Legends

**Figure 1:** LH and cortisol profiles for one representative ewe kept under A) non-stress conditions or B) exposed to the layered stress paradigm on two occasions in experiment 2. The layered stress paradigm is depicted at the top of the panel and consisted of sequential hourly application of isolation, restraint, blindfold and predator cues.

**Figure 2:** Mean pre-stress (closed circles) and immediate post-stress (open and shaded squares) plasma cortisol concentrations in stress ewes in experiment 4 (A, top panel) and experiment 5 (B, bottom panel). The numbers above the post-stress values depict the stressor used: 1) barking dog; 2) circadian stress; 3) layered stress; 4) mock shear; 5) transport; 6) noise/exercise; 7) isolation; and 8) blindfold/barking dog CD. Post-stress values indicated by shaded squares.
denote a significant difference between pre- and post-stress values as determined by paired t-test (P < 0.05), n=6 ewes in experiment 4 and n=8 ewes in experiment 5.

**Figure 3:** Mean daily plasma progesterone values in experiment 4 (A, top panel) for non-stress control (closed circles) and stress ewes (open circles), and in experiment 5 (B, bottom panel) for non-stress control (closed circles), diet only (gray circles) and diet + stress ewes (open circles). The period of stress is depicted at the top of each panel. In experiment 4, values from one stress ewe that expressed an abnormal luteal phase in cycle 3 were excluded. In experiment 5, values were excluded once ewes ceased to express estrous cycles. N=5-6 ewes/group in experiment 4 and n=5-8 ewes/group in experiment 5.

**Figure 4:** Mean daily plasma progesterone profiles of 3 diet only and 2 diet + stress ewes that ceased to express estrous cycles during experiment 5. Sampling ended in Ewe 12 (diet only) after the first missed estrous cycle.

**Figure 5:** Mean estrous cycle parameters in controls (open bars; n=8), diet restricted animals (with or without psychosocial stress) that continued to cycle (hashed bars; n=11) and diet restricted animals that ceased to cycle (closed bars; n=5) in experiment 5. Once an animal displayed disrupted cyclicity, values were excluded from the analysis (ANOVA). * indicates P < 0.02 and ** indicates P < 0.01 compared to control and diet restricted animals that continued to have normal cycles.
Table 1: Effect of Psychosocial Stress on LH Surge Parameters in Experiments 1, 2 and 3

<table>
<thead>
<tr>
<th></th>
<th>n\textsuperscript{a}</th>
<th>Latent Period (h)\textsuperscript{b}</th>
<th>Amplitude (ng/ml)\textsuperscript{c}</th>
<th>Duration (h)\textsuperscript{d}</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Experiment 1</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>40.0 ± 1.3</td>
<td>227.5 ± 19.3</td>
<td>10.6 ± 0.6</td>
</tr>
<tr>
<td>Stress</td>
<td>6</td>
<td>41.8 ± 1.8</td>
<td>227.5 ± 43.3</td>
<td>11.3 ± 0.7</td>
</tr>
<tr>
<td>B. Experiment 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>49.5 ± 0.9</td>
<td>155.8 ± 35.0</td>
<td>13.5 ± 1.9</td>
</tr>
<tr>
<td>Stress</td>
<td>4</td>
<td>49.5 ± 3.6</td>
<td>128.4 ± 21.4</td>
<td>18.0 ± 3.0</td>
</tr>
<tr>
<td>C. Experiment 3</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>4</td>
<td>47.7 ± 1.8</td>
<td>172.8 ± 52.2</td>
<td>9.8 ± 0.8</td>
</tr>
<tr>
<td>Stress</td>
<td>10</td>
<td>45.8 ± 8.6</td>
<td>124.9 ± 17.6</td>
<td>9.9 ± 0.6</td>
</tr>
</tbody>
</table>

\textsuperscript{a} All values are mean ± SEM. No group difference in LH surge latency, amplitude or duration within each experiment as determined by Student’s t-test (P > 0.05); n, number of animals per group.

\textsuperscript{b} Hour from CIDR removal to highest value during LH surge.

\textsuperscript{c} Maximal value during LH surge; pre-surge baseline generally < 3 ng/ml.

\textsuperscript{d} Hour from the surge onset to the time where LH fell to 10% of the surge peak.
Table 2: Effect of Psychosocial Stress on LH Pulse Parameters in Experiment 2

<table>
<thead>
<tr>
<th></th>
<th>Amplitude</th>
<th>Frequency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pre-stress</td>
<td>First Stress Exposure</td>
</tr>
<tr>
<td>Control</td>
<td>1.4 ± 0.2</td>
<td>1.5 ± 0.3</td>
</tr>
<tr>
<td>Stress</td>
<td>2.6 ± 0.4</td>
<td>1.3 ± 0.2</td>
</tr>
</tbody>
</table>

*a All values are mean ± SEM (n=4) across Pre-stress, First and Second Stress Exposure; Amplitude as ng/ml, Frequency as number of pulses / 4 h.

*b Significant treatment x time interaction in repeated measures ANOVA (Pre-stress vs. First Stress Exposure, P < 0.05).
Table 3: Cortisol Values for Experiment 3$^a$

<table>
<thead>
<tr>
<th>Hours from CIDR Removal</th>
<th>Stressor</th>
<th>Pre-Stress$^b$</th>
<th>Stress$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>15 - 18</td>
<td>Food denial, Noise/Exercise</td>
<td>7.2 ± 1.4</td>
<td>17.8 ± 4.1</td>
</tr>
<tr>
<td>22 - 26</td>
<td>Layered stress</td>
<td>14.3 ± 2.9</td>
<td>25.4 ± 9.1$^c$</td>
</tr>
<tr>
<td>31 - 32</td>
<td>Circadian stress</td>
<td>5.9 ± 1.3</td>
<td>37.0 ± 1.3$^c$</td>
</tr>
<tr>
<td>38 - 41</td>
<td>Transport</td>
<td>14.5 ± 2.2</td>
<td>31.3 ± 0.8$^c$</td>
</tr>
<tr>
<td>41 - 42</td>
<td>Mock shear</td>
<td>14.5 ± 2.2</td>
<td>29.9 ± 2.3$^c$</td>
</tr>
</tbody>
</table>

$^a$ All values are mean ± SEM, ng/ml; n=10.
$^b$ Pre-stress values obtained immediately prior to stress initiation; values during stress calculated as mean of samples taken every 20 min until the completion of stress.
$^c$ P < 0.05, pre-stress value vs. value during stress as determined by paired t-test.
Table 4: Effect of Psychosocial Stress on Estrous Cycle Parameters in Experiment 4

<table>
<thead>
<tr>
<th></th>
<th>Follicular/Periovulatory Period&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Luteal Phase Length&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Cycle Length&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Integrated Progesterone&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Peak Progesterone&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 1</td>
<td>5.3 ± 0.3</td>
<td>11.0 ± 0.3</td>
<td>16.8 ± 0.2</td>
<td>29.1 ± 2.3</td>
<td>4.2 ± 0.3</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>3.8 ± 0.3</td>
<td>11.5 ± 0.2</td>
<td>16.8 ± 0.3</td>
<td>35.1 ± 1.9</td>
<td>4.8 ± 0.2</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>4.5 ± 0.4</td>
<td>13.0 ± 0.3</td>
<td>16.8 ± 0.2</td>
<td>41.4 ± 3.0</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>4.5 ± 0.4</td>
<td>12.5 ± 0.2</td>
<td>17.0 ± 0.4</td>
<td>43.2 ± 3.8</td>
<td>5.1 ± 0.4</td>
</tr>
<tr>
<td>Cycle 5</td>
<td>4.2 ± 0.3</td>
<td>12.2 ± 0.2</td>
<td>16.7 ± 0.3</td>
<td>40.0 ± 3.0</td>
<td>5.0 ± 0.3</td>
</tr>
<tr>
<td>Stress</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 1</td>
<td>5.5 ± 0.4</td>
<td>11.0 ± 0.5</td>
<td>17.2 ± 0.5</td>
<td>26.2 ± 1.8</td>
<td>3.7 ± 0.2</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>3.8 ± 0.5</td>
<td>11.5 ± 0.5</td>
<td>17.0 ± 0.5</td>
<td>34.6 ± 3.6</td>
<td>4.7 ± 0.4</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>4.3 ± 0.3</td>
<td>14.7 ± 2.3&lt;sup&gt;d&lt;/sup&gt;</td>
<td>18.7 ± 2.3</td>
<td>45.6 ± 7.9</td>
<td>5.2 ± 0.7</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>4.3 ± 0.4</td>
<td>12.2 ± 0.4</td>
<td>16.5 ± 0.6</td>
<td>37.4 ± 2.2</td>
<td>4.6 ± 0.2</td>
</tr>
<tr>
<td>Cycle 5</td>
<td>4.0 ± 0.3</td>
<td>12.3 ± 0.3</td>
<td>16.7 ± 0.4</td>
<td>37.6 ± 3.7</td>
<td>4.4 ± 0.3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Repeated measures ANOVA revealed no treatment effect or treatment x time interaction in any estrous cycle parameter.

<sup>b</sup> All values are mean ± SEM (n=6); follicular/periovulatory period, luteal phase and cycle length as days; integrated and peak progesterone as ng/ml.

<sup>c</sup> Values during stress cycles are circumscribed by the dashed line.

<sup>d</sup> Mean value includes extremely long luteal phase in one ewe (26 d); mean ± SEM value excluding that ewe is 12.4 ± 0.5 d.
Table 5: Pre-Surge LH Values and LH Surge Parameters for Experiments 4 and 5a

<table>
<thead>
<tr>
<th>Experiment 4, Cycle 4</th>
<th>n</th>
<th>Mean Pre-Surge LHb</th>
<th>Latent Periodb</th>
<th>LH Surge Peak Amplitudeb</th>
<th>LH Surge Durationb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>6</td>
<td>3.0 ± 0.7</td>
<td>46.0 ± 4.0</td>
<td>156.8 ± 27.0</td>
<td>12.0 ± 1.0</td>
</tr>
<tr>
<td>Stress</td>
<td>6</td>
<td>3.1 ± 0.5</td>
<td>48.7 ± 8.9</td>
<td>157.1 ± 23.1</td>
<td>10.7 ± 0.8</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>1.7 ± 0.1</td>
<td>47.6 ± 2.3</td>
<td>191.4 ± 18.7</td>
<td>9.8 ± 0.5</td>
</tr>
<tr>
<td>Diet Only</td>
<td>7</td>
<td>3.0 ± 0.7</td>
<td>48.4 ± 1.7</td>
<td>221.3 ± 24.2</td>
<td>8.6 ± 0.8</td>
</tr>
<tr>
<td>Diet + Stressc</td>
<td>8</td>
<td>2.2 ± 0.3</td>
<td>51.8 ± 3.2</td>
<td>223.9 ± 33.6</td>
<td>10.5 ± 0.6</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>1.9 ± 0.2</td>
<td>50.6 ± 3.0</td>
<td>156.8 ± 17.0</td>
<td>9.0 ± 0.8</td>
</tr>
<tr>
<td>Diet Only</td>
<td>5</td>
<td>1.7 ± 0.2</td>
<td>51.6 ± 4.1</td>
<td>159.7 ± 28.1</td>
<td>10.2 ± 0.7</td>
</tr>
<tr>
<td>Diet + Stressc</td>
<td>7</td>
<td>2.3 ± 0.7</td>
<td>57.9 ± 3.3</td>
<td>199.9 ± 44.3</td>
<td>10.9 ± 0.8</td>
</tr>
</tbody>
</table>

A. Repeated measures ANOVA revealed no treatment x time interaction in any LH parameter.

b All values are mean ± SEM; pre-surge LH and amplitude as ng/ml; latent period as hours from half-maximal progesterone to LH surge peak; duration as hours from surge onset to the time where LH fell to 10% of the surge peak; n, number of animals per group.

c In experiment 5, Cycle 2 was prior to and Cycle 4 during the psychosocial stress period.
Table 6: Effect of Psychosocial Stress on Estrous Cycle Parameters in Experiment 5a

<table>
<thead>
<tr>
<th></th>
<th>Follicular/Periovulatory Periodb</th>
<th>Luteal Phase Lengthb</th>
<th>Cycle Lengthb</th>
<th>Integrated Progesteroneb</th>
<th>Peak Progesteroneb</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 1</td>
<td>4.1 ± 0.2</td>
<td>12.5 ± 0.3</td>
<td>15.5 ± 0.2</td>
<td>42.1 ± 3.3</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>3.3 ± 0.3</td>
<td>12.4 ± 0.4</td>
<td>16.6 ± 0.3</td>
<td>44.1 ± 2.9</td>
<td>5.6 ± 0.3</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>3.3 ± 0.4</td>
<td>13.3 ± 0.3</td>
<td>16.5 ± 0.3</td>
<td>57.6 ± 2.2</td>
<td>7.0 ± 0.3</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>3.5 ± 0.3</td>
<td>13.3 ± 0.3</td>
<td>16.4 ± 0.3</td>
<td>57.5 ± 3.6</td>
<td>7.1 ± 0.4</td>
</tr>
<tr>
<td><strong>Diet Only</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 1</td>
<td>3.6 ± 0.2</td>
<td>12.0 ± 0.3</td>
<td>15.6 ± 0.3</td>
<td>39.7 ± 3.5</td>
<td>5.3 ± 0.4</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>4.7 ± 1.1</td>
<td>13.3 ± 0.2</td>
<td>16.9 ± 0.3</td>
<td>44.2 ± 3.1</td>
<td>5.2 ± 0.4</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>2.8 ± 0.2</td>
<td>12.9 ± 0.4</td>
<td>17.6 ± 0.8</td>
<td>48.2 ± 5.2</td>
<td>5.9 ± 0.6</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>4.2 ± 0.3</td>
<td>13.8 ± 0.2</td>
<td>17.0 ± 0.3</td>
<td>59.8 ± 5.2</td>
<td>7.4 ± 0.6</td>
</tr>
<tr>
<td><strong>Diet + Stress</strong></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Cycle 1</td>
<td>3.9 ± 0.3</td>
<td>12.1 ± 0.3</td>
<td>15.1 ± 0.4</td>
<td>43.5 ± 5.0</td>
<td>5.4 ± 0.6</td>
</tr>
<tr>
<td>Cycle 2</td>
<td>4.0 ± 0.7</td>
<td>13.1 ± 0.4</td>
<td>17.0 ± 0.3</td>
<td>46.5 ± 4.7</td>
<td>5.2 ± 0.5</td>
</tr>
<tr>
<td>Cycle 3</td>
<td>3.5 ± 0.4</td>
<td>13.3 ± 0.7</td>
<td>17.4 ± 0.5</td>
<td>57.9 ± 6.5</td>
<td>6.8 ± 0.6</td>
</tr>
<tr>
<td>Cycle 4</td>
<td>4.2 ± 0.3</td>
<td>13.0 ± 0.3</td>
<td>16.4 ± 0.3</td>
<td>53.5 ± 6.1</td>
<td>6.6 ± 0.8</td>
</tr>
</tbody>
</table>

a Repeated measures ANOVA revealed no treatment effect or time x treatment interaction in any estrous cycle parameter. Values include only cycling ewes.

b All values are mean ± SEM; follicular/periovulatory period, luteal phase and cycle length as days; integrated and peak progesterone as ng/ml.

c Values during stress cycles are circumscribed by the dashed line.
Figure 1: Representative Profiles, Experiment 2

A. Non-stress control

B. Stress

Cortisol (ng/ml)
LH (ng/ml)

Hour from CIDR Removal
Figure 2: Mean Stress-Induced Cortisol Values, Experiments 4 and 5

A. Experiment 4

B. Experiment 5
Figure 3: Mean Daily Progesterone Values, Experiments 4 and 5

A. Experiment 4

B. Experiment 5

Figure 3, Wagenmaker et al.
Figure 4: Daily Progesterone Values for Ewes with Disrupted Cycles, Experiment 5

![Graph showing daily progesterone values for different ewes. The graphs represent Ewe 11 Diet Only, Ewe 12 Diet Only, Ewe 23 Diet Only, Ewe 14 Diet + Stress, and Ewe 18 Diet + Stress. The x-axis represents days from CIDR removal, and the y-axis represents progesterone levels (ng/ml).]
Figure 5, Wagenmaker et al.

Plasma progesterone (ng/ml)

Peak

- Control
- Diet not disrupted
- Diet disrupted

Integrated

Length of cycle stages (days)

Luteal phase

- Control
- Diet not disrupted
- Diet disrupted

Follicular/ periovulatory

- Control
- Diet not disrupted
- Diet disrupted

Entire cycle

- Control
- Diet not disrupted
- Diet disrupted