
Brief Report

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

Melissa E. Lenert, Micaela M. Chaparro, and Michael D. Burton

Neuroimmunology and Behavior Group, Department of Neuroscience, School of Behavioral and Brain Sciences, Center for Advanced Pain Studies, University of Texas at Dallas, Richardson, TX 75080, USA

ORCID numbers: 0000-0002-5504-4700 (M. E. Lenert); 0000-0001-8098-414X (M. M. Chaparro); 0000-0002-0628-824X (M. D. Burton).

Abbreviations: PCOS, polycystic ovary syndrome; SEM, standard error of the mean.

Received: 7 July 2020; Editorial Decision: 25 January 2021; First Published Online: 1 February 2021; Corrected and Typeset: 10 March 2021.

Abstract

The etiology of reproductive disorders correlates 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 that female mice are resistant to high-fat diet–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 high-fat diet. However, reproductive changes that occur in adolescent mice on high-fat diet 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 high-fat diet. Female mice were put on high-fat diet or control diet for 12 weeks starting at 4 weeks of age. Every 4 weeks, their estrus cycle was tracked and fasting glucose was measured. We found that after 4 weeks on high-fat diet, there was no difference in weight between groups, but an increase in time spent in proestrus and estrus in mice on high-fat diet and an increase in serum progesterone during proestrus. These results show that intact females modulate their estrus cycle in response to a high-fat diet 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.

Key Words: high-fat diet, females, estrus cycle, metabolism, reproduction, adolescence

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 4 weeks of age, females were given either a control diet or a 45% high-fat diet, and estrus cycling was monitored for 12 weeks on diet. As expected, there were no differences in weight gain or glucose metabolism. We show that estrus cycling is altered at 4, but not 8, 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 4, but not 8, 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

Animals

Young adult female C57BL/6J mice were used for experiments. Mice were weaned at 3 weeks of age and put onto assigned diet (high-fat diet or control chow diet) starting at 4 weeks of age for up to 12 weeks. The onset of sexual maturity served as the beginning of the diet change and persisted for 8 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 12-hour light-dark cycle (lights on from 06:00h to 18:00h) 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 4 weeks of diet, up to 12 weeks. For the high-fat diet group, there were 14 animals through week 4, 10 through 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 16:00h and 18:00h for 12 weeks. Fasting glucose levels were tested once every 4 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 08:00h. 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 09:00h and once at 18:00h, as described previously [21-23]. Mice were sampled for 9 days every 4 weeks for up to 12 weeks on diet. Vaginal lavage was performed by flushing the vagina with 20 μ L 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

2 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 1 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 5× and 20× magnification.

Estrus cycle phases were classified by the cytology described in Cora et al (2015) [23]. Briefly, proestrus (Fig. 1a and 1b) 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 to 24 hours. Estrus (Fig. 1c and 1d) 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 (Fig. 1e and 1f) 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 to 24 hours. Additionally, smears classified as metestrus contain the highest cell density of all the phases. Diestrus (Fig. 1g and 1h) 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 to 72 hours.

Smears were classified in a randomized order such that the experimenter was blinded to both time and diet. Values for Fig. 3 were calculated by dividing the number of smears in each phase by the total number of samples for each mouse (ie, 2 smears in proestrus / 20 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 high-fat and control diet were imaged at 10× magnification. Each cell type was manually counted using ImageJ software within the entire imaged area. The mean \pm standard error of the mean (SEM)

number of cells counted in smears across the cycle can be found in Table 2.

Progesterone Enzyme-Linked Immunosorbent Assay

Serum was isolated from tail blood collected between 09:00h and 11:00h, immediately following vaginal smear collection, for 9-day sampling periods every 4 weeks (Fig. 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 15 000 rpm at 4 °C for 15 minutes. Samples were immediately stored at -80 °C until the 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 to 100 ng/mL and sensitivity of 0.156 ng/mL. Mean intra-assay and inter-assay coefficient of variation 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 \pm SEM. Weight gain, fasting glucose, estrus cycle, and serum progesterone concentration data were 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 4 and 8 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 Fig. 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 12 weeks (Fig. 2b). There was no

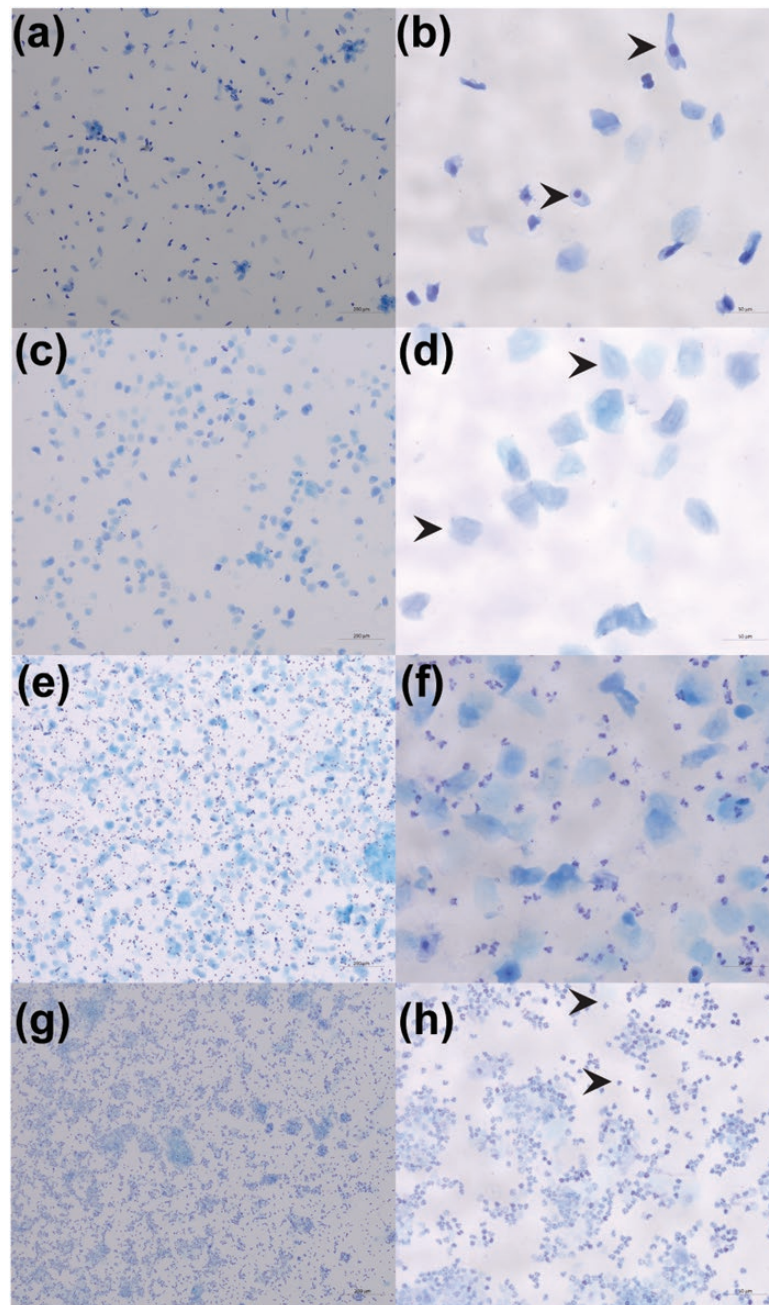


Figure 1. Representative vaginal smears from each phase of the estrus cycle. Representative vaginal smears from proestrus (a, b), estrus (c, d), metestrus (e, f), and diestrus (g, h) stained with Toluidine Blue O. Proestrus is characterized by nucleated epithelial cells (b, arrows). Estrus is characterized 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).

difference in weights or fasting glucose at the start of diet treatment. Mice were weighed weekly between 17:00h and 18:00h, just prior to the start of the dark cycle. All mice gained weight over the 12-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 4 weeks (Fig. 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

Table 1. Estrus Cycle Tracking of Female Mice On High-Fat Diet for 12 Weeks

		High Fat Diet										Control																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																							
		Cohort 1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
Day	Time	Week 0								Week 0																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
1	PM	D	-	M	M	-	D	-	-	D	M	-	D	-	-	D	D		AM	-	D	D	D	-	-	-	-	-	-	D	M	D	D	-	-	2	PM	D	D	D	-	D	D	D	-	-	M	-	D	-	D	-	D		AM	-	-	P*	D	-	-	D	D	-	-	D	D	E*	P*	-	D	3	PM	-	-	-	-	D	D	D	P*	-	-	D	-	D	P	D	P*		AM	-	-	D	D	D	D	D	E	M	-	D	P*	P	E	D	E	4	PM	-	-	D	D	E*	E*	D	E	-	-	-	-	E	M	P*	D		AM	-	D	D	P*	-	-	M	D	D	D	-	E	P	D	E	D	5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D
	AM	-	D	D	D	-	-	-	-	-	-	D	M	D	D	-	-	2	PM	D	D	D	-	D	D	D	-	-	M	-	D	-	D	-	D		AM	-	-	P*	D	-	-	D	D	-	-	D	D	E*	P*	-	D	3	PM	-	-	-	-	D	D	D	P*	-	-	D	-	D	P	D	P*		AM	-	-	D	D	D	D	D	E	M	-	D	P*	P	E	D	E	4	PM	-	-	D	D	E*	E*	D	E	-	-	-	-	E	M	P*	D		AM	-	D	D	P*	-	-	M	D	D	D	-	E	P	D	E	D	5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																		
2	PM	D	D	D	-	D	D	D	-	-	M	-	D	-	D	-	D		AM	-	-	P*	D	-	-	D	D	-	-	D	D	E*	P*	-	D	3	PM	-	-	-	-	D	D	D	P*	-	-	D	-	D	P	D	P*		AM	-	-	D	D	D	D	D	E	M	-	D	P*	P	E	D	E	4	PM	-	-	D	D	E*	E*	D	E	-	-	-	-	E	M	P*	D		AM	-	D	D	P*	-	-	M	D	D	D	-	E	P	D	E	D	5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																				
	AM	-	-	P*	D	-	-	D	D	-	-	D	D	E*	P*	-	D	3	PM	-	-	-	-	D	D	D	P*	-	-	D	-	D	P	D	P*		AM	-	-	D	D	D	D	D	E	M	-	D	P*	P	E	D	E	4	PM	-	-	D	D	E*	E*	D	E	-	-	-	-	E	M	P*	D		AM	-	D	D	P*	-	-	M	D	D	D	-	E	P	D	E	D	5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																						
3	PM	-	-	-	-	D	D	D	P*	-	-	D	-	D	P	D	P*		AM	-	-	D	D	D	D	D	E	M	-	D	P*	P	E	D	E	4	PM	-	-	D	D	E*	E*	D	E	-	-	-	-	E	M	P*	D		AM	-	D	D	P*	-	-	M	D	D	D	-	E	P	D	E	D	5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																								
	AM	-	-	D	D	D	D	D	E	M	-	D	P*	P	E	D	E	4	PM	-	-	D	D	E*	E*	D	E	-	-	-	-	E	M	P*	D		AM	-	D	D	P*	-	-	M	D	D	D	-	E	P	D	E	D	5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																										
4	PM	-	-	D	D	E*	E*	D	E	-	-	-	-	E	M	P*	D		AM	-	D	D	P*	-	-	M	D	D	D	-	E	P	D	E	D	5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																												
	AM	-	D	D	P*	-	-	M	D	D	D	-	E	P	D	E	D	5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																														
5	PM	-	-	D	P	-	E	-	D	-	-	-	E	P	D	E	D		AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																
	AM	D	D	D	-	D	D	D	D	M	-	D	D	D	D	E	-	6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																		
6	PM	D	-	D	E	D	P	E*	D	M	-	D	D	D	D	M	-		AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																				
	AM	-	D	D	E	D	D	D	D	D	D	-	P	E	D	D	D	7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																						
7	PM	-	-	D	E	D	P	P	D	D	D	-	P	E	D	D	D		AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																								
	AM	D	D	D	M	-	-	D	D	-	-	D	E	-	-	D	D	8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																										
8	PM	E*	E*	D	D	P	M	M	D	-	-	D	E	E	-	D	E		AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																												
	AM	D	D	D	D	E	M	D	D	E*	M	D	E	M	-	D	E	9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																														
9	PM	D	-	D	D	M	-	M	P	E	D	D	M	M	D	P	E		AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E			Cohort 2																Day	Time	Week 0								Week 0								1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																
	AM	D	E	D	D	D	P	M	D	E	D	D	D	E	D	D	E																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
		Cohort 2																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
Day	Time	Week 0								Week 0																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
1	PM	D	D	D	M	E*	M	-	D	D	D	D	D	D	D	D	D		AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																								
	AM	-	D	D	M	E	D	-	-	-	D	D	D	D	D	D	D	2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																										
2	PM	-	D	D	D	M	D	D	-	D	D	D	D	D	D	D	D		AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																												
	AM	D	D	D	D	M	D	-	D	D	D	D	D	D	D	D	D	3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																														
3	PM	E*	D	D	P*	M	D	D	D	D	M	P*	D	D	D	D	D		AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																
	AM	E	E*	D	E	M	D	E*	D	D	D	E	D	D	D	D	D	4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
4	PM	D	E	P*	E	D	D	E	P*	D	E	D	D	D	D	D	D		AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
	AM	D	E	E	E	D	D	E	E	D	D	D	D	D	D	D	D	5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
5	PM	P	M	D	M	D	D	M	D	D	D	D	D	D	D	D	D		AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
	AM	E	D	D	D	D	D	D	D	D	M	D	D	D	D	D	D	6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																										
6	PM	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D	D		AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
	AM	D	D	D	D	D	D	-	D	D	D	E	D	D	D	D	D	7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
7	PM	-	D	D	P	P	D	-	P	D	E	D	D	D	D	D	D		AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
	AM	D	D	E	M	M	D	D	E	E*	M	D	D	D	D	D	D	8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
8	PM	D	P	E	D	D	P*	D	E	M	D	D	D	D	D	D	D		AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
	AM	E	E	D	D	D	E	D	M	D	E	D	D	D	D	D	D	9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
9	PM	E	E	D	D	D	E	D	D	D	D	E	D	D	D	D	D		AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D			Cohort 1																Day	Time	Week 4								Week 4								1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
	AM	E	D	D	D	D	E	-	D	D	E	D	D	D	D	D	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
		Cohort 1																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																	
Day	Time	Week 4								Week 4																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																									
1	PM	E	M	M	M	E	P	E	M	D	M	D	M	P	M	E	E		AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
	AM	E	D	E	D	E	E	E	M	M	D	P	M	E	D	M	M	2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		
2	PM	E	D	E	D	D	E	M	E	M	D	P	D	E	D	M	P		AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																				
	AM	E	D	E	D	D	E	M	P	M	D	E	P	M	D	D	D	3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																						
3	PM	D	D	E	P	D	E	M	E	D	D	E	E	M	D	P	D		AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								
	AM	P	D	D	E	-	-	-	E	D	D	E	D	D	M	E	D	4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																										
4	PM	D	D	D	M	D	D	D	M	D	P	M	E	D	M	E	D		AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																												
	AM	D	D	D	E	-	-	-	D	P	D	M	E	D	M	M	D	5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																														
5	PM	D	D	D	E	D	D	D	P	E	D	D	P	D	D	M	D		AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																
	AM	D	D	D	E	D	D	D	E	E	D	D	E	D	D	M	D																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																		

Table 1. Continued

		High-fat diet								Control							
		Cohort 1															
Day	Time	Week 8								Week 8							
6	PM	E	E	D	E	D	D	D	E	E	D	P	E	D	D	M	D
	AM	E	E	D	E	P	D	D	E	E	D	E	D	D	P	M	D
7	PM	E	E	P	M	E	P	P	E	E	D	M	D	P	P	P	D
	AM	M	E	E	D	E	E	E	D	M	D	D	D	E	E	D	E
8	PM	D	E	E	D	E	E	E	D	D	D	D	D	E	E	D	E
	AM	D	D	E	D	M	M	M	D	D	D	D	D	M	E	D	E
9	PM	D	D	E	P	D	D	D	P	D	D	D	D	D	D	D	M
	AM	P	D	D	P	D	D	D	E	D	D	D	D	D	D	D	D
		Cohort 2															
Day	Time	Week 4								Week 4							
1	PM	E	P	D	M	D	P			P	P	P	E				
	AM	M	D	M	D	D	E			E	D	E	E				
2	PM	M	M	E	D	P	D			D	E	M	E				
	AM	D	P	D	D	P	D			E	D	D	M				
3	PM	D	E	D	E	P	D			E	D	D	D				
	AM	D	E	D	E	E	D			M	D	D	D				
4	PM	D	E	D	E	M	P			D	D	D	D				
	AM	P	M	D	E	E	E			P	D	D	D				
5	PM	E	D	D	E	E	M			P	D	E	D				
	AM	M	D	E	D	E	D			D	D	E	D				
6	PM	M	D	M	D	M	D			P	D	M	D				
	AM	M	D	D	D	E	D			E	D	D	D				
7	PM	M	P	D	D	D	D			E	P	D	D				
	AM	D	E	D	P	D	D			M	E	D	D				
8	PM	D	E	P	P	D	D			D	E	P	M				
	AM	D	E	E	P	D	D			D	D	E	D				
9	PM	D	M	E	P	D	D			D	D	E	M				
	AM	E	D	E	E	D	P			D	D	E	D				
		Cohort 1															
Day	Time	Week 8								Week 8							
1	PM	E	D	E	D	E	D			D	E	D	M				
	AM	P	P	P	M	M	D			P	E	M	E				
2	PM	P	P	E	D	D	D			P	E	P	D				
	AM	E	E	E	D	D	D			E	E	E	D				
3	PM	-	E	M	D	D	P			-	-	E	D				
	AM	E	-	D	D	P	E			E	E	E	D				
4	PM	E	E	P	D	E	E			E	-	M	D				
	AM	E	E	P	D	E	E			E	M	D	D				
5	PM	E	M	D	D	M	E			M	D	D	E				
	AM	M	D	P	D	M	E			D	D	D	M				
6	PM	D	D	E	D	E	E			D	D	D	D				
	AM	-	D	E	D	E	E			D	D	D	D				
7	PM	D	D	E	D	E	M			P	P	D	D				
	AM	D	D	M	D	M	D			E	E	D	D				
8	PM	D	P	D	D	M	D			E	E	D	E				
	AM	P	E	D	D	D	M			E	E	D	M				
9	PM	E	E	D	D	D	P			E	M	P	D				
	AM	E	E	D	D	D	E			M	D	E	D				

Table 1. Continued

		High-fat diet					Control				
		Cohort 2									
Day	Time	Week 8					Week 8				
1	PM	E	E	D	D	E	E	E	D		
	AM	E	E	D	M	E	E	E	E		
2	PM	M	M	D	D	M	E	E	M		
	AM	P	E	D	M	M	E	E	M		
3	PM	M	E	D	M	D	M	M	M		
	AM	M	E	P	D	D	D	D	M		
4	PM	D	E	E	M	P	P	D	D		
	AM	D	E	E	M	E	E	D	D		
5	PM	P	P	M	E	E	E	D	E		
	AM	E	P	P	E	M	M	P	E		
6	PM	E	P	E	M	D	D	E	E		
	AM	E	E	E	D	P	P	E	E		
7	PM	D	E	M	D	E	P	E	E		
	AM	D	E	D	E	M	E	E	D		
8	PM	P	E	D	M	D	M	M	P		
	AM	P	M	P	P	D	M	D	D		
9	PM	E	D	E	E	P	M	D	P		
	AM	E	E	E	E	E	M	D	E		
Cohort 1											
Day	Time	Week 12					Week 12				
1	PM	M	M	D	E	D	D	E	E		
	AM	D	D	D	D	P	D	P	P		
2	PM	D	D	E	P	E	D	E	D		
	AM	M	M	E	E	E	M	E	D		
3	PM	M	D	E	E	E	D	E	D		
	AM	D	D	E	E	E	D	E	D		
4	PM	D	D	E	M	D	D	M	D		
	AM	D	D	E	D	D	D	D	D		
5	PM	D	D	E	D	D	D	D	P		
	AM	P	P	D	D	D	D	D	E		
6	PM	E	P	D	P	E	P	D	E		
	AM	E	E	D	E	E	E	D	E		
7	PM	E	E	D	E	E	E	D	M		
	AM	E	E	D	E	D	E	D	D		
8	PM	M	D	M	M	P	M	D	D		
	AM	D	D	P	D	P	D	E	E		
9	PM	D	D	E	E	E	D	E	D		
	AM	D	D	D	D	E	D	D	D		

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. Abbreviations: D, diestrus; E, estrus; M, metestrus; P, proestrus. *indicates first appearance of anucleated epithelial cells.

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 (Fig. 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 4 weeks of diet treatment (Fig. 2e). There was no significant difference in cycle length at any

Table 2. Quantification of Cell Populations Across the Estrus Cycle

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

Data are mean (± SEM) number of cells in Toluidine Blue O-stained vaginal smears. n = 5 per phase. Samples were quantified from mice of both diets.

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 (Fig. 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 4 weeks of age. Estrus cycles were tracked for 9 days, twice per day, starting every 4 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 4.5 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 4 out of 14 animals in the high-fat diet group and 3 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 5 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 (Fig. 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 (Fig. 3b). After consuming a high-fat diet for 4 weeks, there was a significant increase in the amount of time spent in proestrus ($P = 0.0012$, compared with control) and in estrus for mice on high-fat diet ($P = 0.0287$, compared with control). By week 8 of assigned 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 (Fig. 3c) or diestrus (Fig. 3d) at any of the time points measured; however, there was approximately a 10% decrease in time spent in diestrus at 4 weeks on high-fat diet compared with control. This decrease was not statistically significant ($P = 0.0793$, high-fat diet 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 4 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 8 or 12 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 4 of high-fat diet, we assessed serum progesterone levels across the cycle (proestrus, estrus, and diestrus) every 4 weeks on high-fat diet. The standard curve had a R^2 value of 0.9979. During proestrus (Fig. 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 × diet: $F = 36.07$, $P < 0.0001$). There was an increase in serum progesterone at 4 weeks on diet, corresponding to the increased time in proestrus at this timepoint. During estrus (Fig. 3f), there was a significant effect of diet on serum progesterone levels (diet: $F = 8.292$, $P = 0.0093$) and a significant interaction (time × diet: $F = 6.905$, $P = 0.0023$); however, we did not see differences in serum progesterone levels at 4 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 12-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 report here the novel finding that alterations in estrus cycling occur in female C57BL/6J wild-type mice at 4 weeks on a high-fat diet without changes in weight

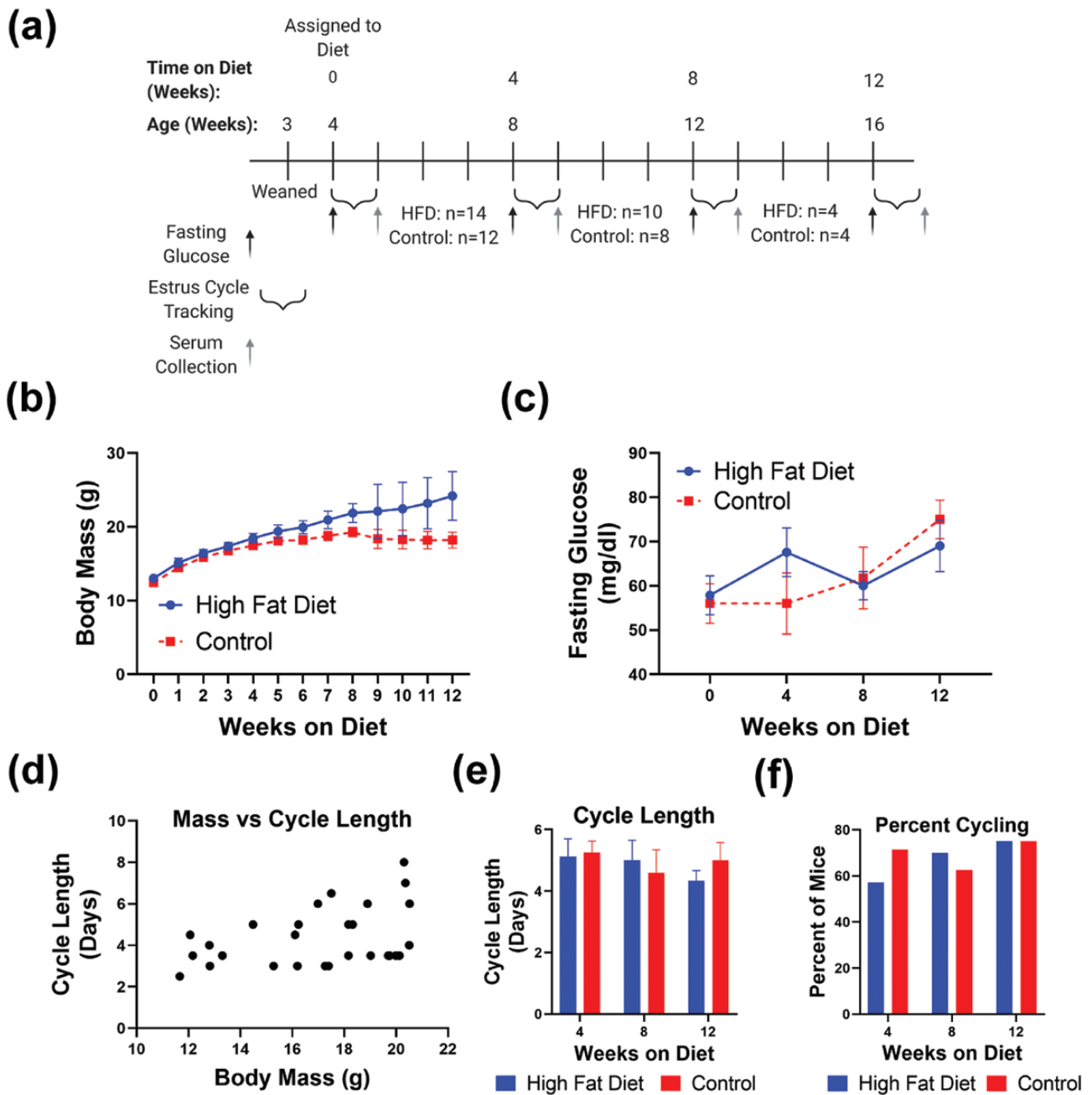


Figure 2. Female C57BL/6J mice are initially resistant to high-fat diet-induced weight gain. (a) Representation of the experimental timeline. (b) Body mass (mean $g \pm$ SEM) of female mice fed either a high-fat diet or control chow diet for 12 weeks. Diet was assigned at 4 weeks of age. All mice gained 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 (\pm SEM) fasting glucose levels measured at every 4 weeks on high-fat diet. High-fat diet: blue, solid line; Weeks 0 to 4: $n = 14$, Weeks 5 to 8: $n = 10$, Weeks 9 to 12: $n = 4$. Control diet: red, dashed line; Weeks 0 to 4: $n = 12$, Weeks 4 to 8: $n = 8$, Weeks 9 to 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 1 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 (\pm SEM) to complete 1 estrus cycle during each sampling period. Acyclic mice were excluded from this analysis. There is no effect of diet on rates of acyclicity (f). Values are the percentage of mice with at least 1 complete estrus cycle during each sampling period.

and glucose metabolism. Interestingly, we also show that estrus cycling returns to normal at 8 weeks on the 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].

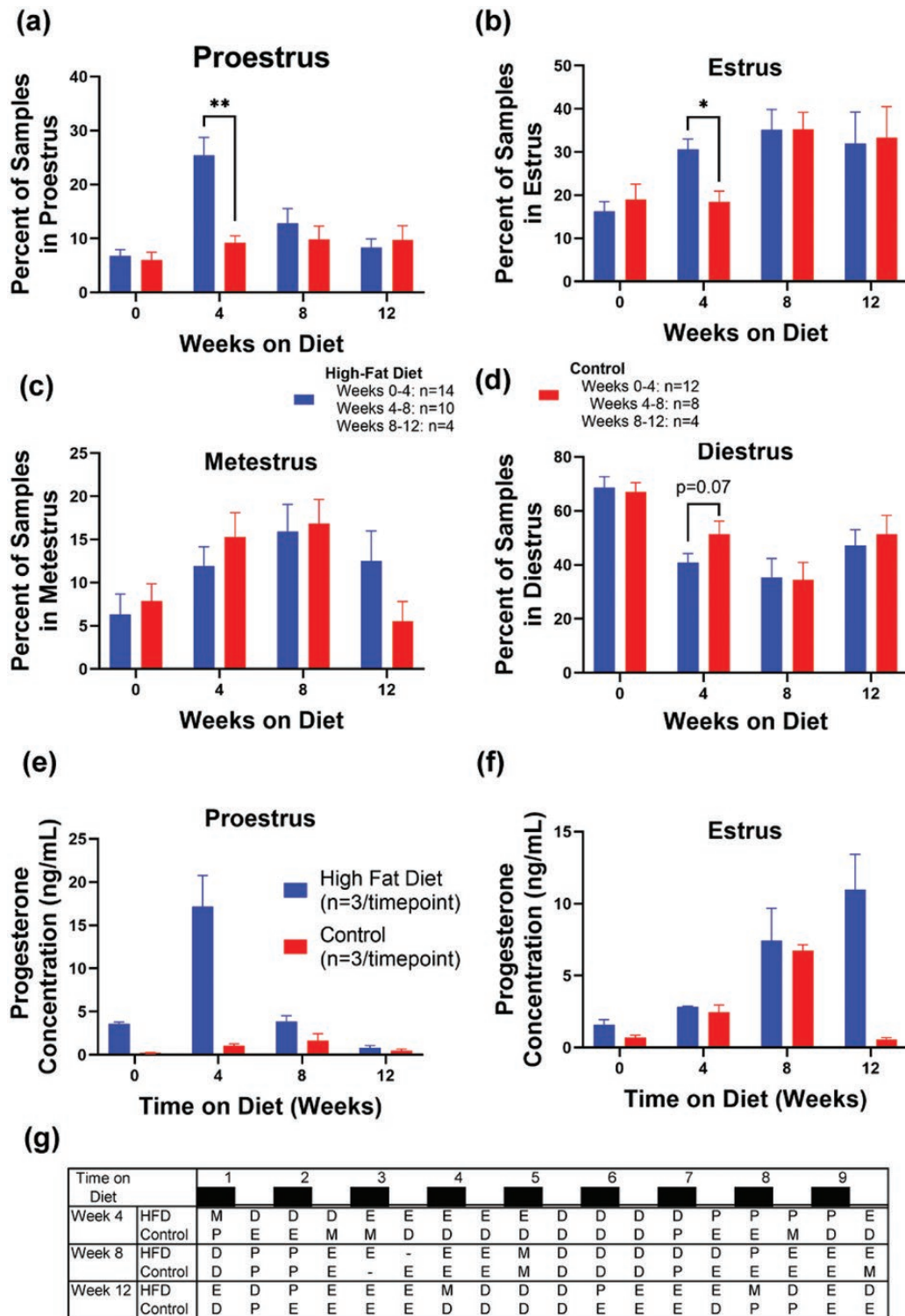


Figure 3. Short-term high-fat diet causes changes in estrus cycling patterns and serum progesterone levels. Representation of proportion of samples (mean number of samples ± SEM) spent in proestrus (a), estrus (b), metestrus (c), and diestrus (d) measured over 9 days every 4 weeks on high-fat diet. For 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 4 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 4 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 enzyme-linked immunosorbent assay. (e) There is an increase in progesterone during proestrus at 4 weeks on high-fat diet but at no other timepoints. (f) There is an increase in progesterone during estrus at 12 weeks on high-fat diet but at no other timepoints. $n = 3/\text{diet}$ for each timepoint. High-fat diet: blue; Weeks 0 to 4: $n = 14$, Weeks 5 to 8: $n = 10$, Weeks 9 to 12: $n = 4$. Control diet: red; Weeks 0 to 4: $n = 12$, Weeks 4 to 8: $n = 8$, Weeks 9 to 12: $n = 4$. *indicates comparison to control diet. (g) Representative cycles from mice on high-fat and control diet at each timepoint. P = proestrus. E = estrus. M = metestrus. D = diestrus. Shading indicates samples collected during the dark cycle.

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 a 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 4, we also saw a decrease in the time spent in diestrus, although not statistically significant. Taken together, increased time spent in proestrus and estrus and decreased time spent in diestrus point toward increased fertility potential in mice on acute high-fat diet. The disappearance of this enhanced proestrus phase by 8 weeks on diet shows that the 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 12-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 2 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 were 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 in future studies 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.

Acknowledgments

The authors would like to thank Natalia Lucia dos Santos for her technical assistance in cycle collection and analysis. We would also like to thank all current and former lab members for the assistance and input on this manuscript. Graphics created with [Biorender.com](https://biorender.com).

Financial Support: This research was funded by the National Institutes of Health/National Institute of Neurological Disorders and Stroke, grant number K22NS096030 (M.D.B.), the University of Texas System STARS program research support grant (M.D.B.), the American Pain Society Future Leaders Grant (M.D.B.), and the Rita Allen Foundation Grant (M.D.B.).

Additional Information

Correspondence: Michael D. Burton, PhD, Department of Neuroscience, School of Behavioral and Brain Sciences, University of Texas at Dallas, 800 W Campbell Rd, Richardson, TX 75080, USA. Email: Michael.Burton@utdallas.edu.

Disclosures: The authors have nothing to disclose.

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.

References

1. Wuttke W. Neuroendocrine mechanisms in reproductive physiology. *Rev Physiol Biochem Pharmacol.* 1976;76:59-101.
2. Hill JW, Elmquist JK, Elias CF. Hypothalamic pathways linking energy balance and reproduction. *Am J Physiol Endocrinol Metab.* 2008;294(5):E827-E832.
3. Colledge WH. The neuroendocrine regulation of the mammalian reproductive axis. Introduction. *Exp Physiol.* 2013;98(11):1519-1521.
4. Lainez NM, Coss D. Obesity, neuroinflammation, and reproductive function. *Endocrinology.* 2019;160(11):2719-2736.

5. Mikhael S, Punjala-Patel A, and Gavrilova-Jordan L., Hypothalamic-pituitary-ovarian axis disorders impacting female fertility. *Biomedicines*, 2019. 7(1).
6. Ruddenklau A, Campbell RE. Neuroendocrine impairments of polycystic ovary syndrome. *Endocrinology*. 2019;160(10):2230-2242.
7. Witchel SF, Oberfield SE, Peña AS. Polycystic ovary syndrome: pathophysiology, presentation, and treatment with emphasis on adolescent girls. *J Endocr Soc*. 2019;3(8):1545-1573.
8. Ibáñez L, Oberfield SE, Witchel S, et al. An international consortium update: pathophysiology, diagnosis, and treatment of polycystic ovarian syndrome in adolescence. *Horm Res Paediatr*. 2017;88(6):371-395.
9. Witchel SF, et al., The diagnosis of polycystic ovary syndrome during adolescence. *Hormone Research in Paediatrics* 2015. 83(6): 376-389.
10. Perrigo G, Bronson FH. Foraging effort, food intake, fat deposition and puberty in female mice. *Biol Reprod*. 1983;29(2):455-463.
11. Robker RL, Wu LL, Yang X. Inflammatory pathways linking obesity and ovarian dysfunction. *J Reprod Immunol*. 2011;88(2):142-148.
12. Sirotkin AV, Fabian D, Babelová Kubandová J, Vlčková R, Alwaseel S, Harrath AH. Body fat affects mouse reproduction, ovarian hormone release, and response to follicular stimulating hormone. *Reprod Biol*. 2018;18(1):5-11.
13. Evans MC, Anderson GM. Integration of Circadian and Metabolic Control of Reproductive Function. *Endocrinology*. 2018;159(11):3661-3673.
14. Lainez NM, Jonak CR, Nair MG, et al. Diet-induced obesity elicits macrophage infiltration and reduction in spine density in the hypothalamus of male but not female mice. *Front Immunol*. 2018;9:1992.
15. Hwang LL, Wang CH, Li TL, et al. Sex differences in high-fat diet-induced obesity, metabolic alterations and learning, and synaptic plasticity deficits in mice. *Obesity (Silver Spring)*. 2010;18(3):463-469.
16. Martin B, Pearson M, Kebejian L, et al. Sex-dependent metabolic, neuroendocrine, and cognitive responses to dietary energy restriction and excess. *Endocrinology*. 2007;148(9):4318-4333.
17. López M, Tena-Sempere M. Estrogens and the control of energy homeostasis: a brain perspective. *Trends Endocrinol Metab*. 2015;26(8):411-421.
18. Palmisano BT, Stafford JM, Pendergast JS. High-Fat Feeding Does Not Disrupt Daily Rhythms in Female Mice because of Protection by Ovarian Hormones. *Front Endocrinol (Lausanne)*. 2017;8:44.
19. Bryzgalova G, Lundholm L, Portwood N, et al. Mechanisms of antidiabetogenic and body weight-lowering effects of estrogen in high-fat diet-fed mice. *Am J Physiol Endocrinol Metab*. 2008;295(4):E904-E912.
20. Kubota K, Cui W, Dhakal P, et al. Rethinking progesterone regulation of female reproductive cyclicity. *Proc Natl Acad Sci U S A*. 2016;113(15):4212-4217.
21. Ajayi AF, Akhigbe RE. Staging of the estrous cycle and induction of estrus in experimental rodents: an update. *Fertil Res Pract*. 2020;6:5.
22. Byers SL, Wiles MV, Dunn SL, Taft RA. Mouse estrous cycle identification tool and images. *Plos One*. 2012;7(4):e35538.
23. Cora MC, Kooistra L, Travlos G. Vaginal cytology of the laboratory rat and mouse: review and criteria for the staging of the estrous cycle using stained vaginal smears. *Toxicol Pathol*. 2015;43(6):776-793.
24. Caligioni CS. Assessing reproductive status/stages in mice. *Curr Protoc Neurosci*. 2009;48(1):A.41.1-A41.8.
25. Goldman JM, Murr AS, Cooper RL. The rodent estrous cycle: characterization of vaginal cytology and its utility in toxicological studies. *Birth Defects Res B Dev Reprod Toxicol*. 2007;80(2):84-97.
26. Hubscher CH, Brooks DL, Johnson JR. A quantitative method for assessing stages of the rat estrous cycle. *Biotech Histochem*. 2005;80(2):79-87.
27. Heinecke H, Grimm H. Studies on the opening time of the vaginal membranes in various mice strains. *Endokrinologie*. 1958;35(4):205-213.
28. Rudali G, Juliard L, Yourkovski N, Fautrel M. Time of the sexual maturation of various strains of mouse, with special reference to the NLC strain. *J Physiol (Paris)*. 1957;49(3):657-666.
29. McLean AC, et al., Performing vaginal lavage, crystal violet staining, and vaginal cytological evaluation for mouse estrous cycle staging identification. *J Vis Exp*. 2012(67):e4389.