

RESEARCH ARTICLE

Pregnancy stage determines the effect of chronic stress on ovarian progesterone synthesis

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Submitted 9 May 2018; accepted in final form 8 August 2018

Wilsterman K, Gotlieb N, Kriegsfeld LJ, Bentley GE. Pregnancy stage determines the effect of chronic stress on ovarian progesterone synthesis. *Am J Physiol Endocrinol Metab* 315: E987–E994, 2018. First published August 14, 2018; doi:10.1152/ajpendo.00183.2018.—Although stress-induced glucocorticoid release is thought to be a primary driver by which maternal stress negatively impacts pregnancy outcomes, the downstream neuroendocrine targets mediating these adverse outcomes are less well understood. We hypothesized that stress-induced glucocorticoid secretion inhibits pituitary hormone secretion, resulting in decreased ovarian progesterone synthesis. Using a chronic restraint model of stress in mice, we quantified steroid hormone production, pituitary hormones, and expression of ovarian genes that support progesterone production at both early (*day 5*) and midpregnancy (*day 10*). Females subjected to daily restraint had elevated baseline glucocorticoids during both early and midpregnancy; however, lower circulating progesterone was observed only during early pregnancy. Lower progesterone production was associated with lower expression of steroidogenic enzymes in the ovary of restrained females during early pregnancy. There were no stress-related changes to luteinizing hormone (LH) or prolactin (PRL). By midpregnancy, circulating LH decreased regardless of treatment, and this was associated with downregulation of ovarian steroidogenic gene expression. Our results are consistent with a role for LH in maintaining steroidogenic enzyme expression in the ovary, but neither circulating PRL nor LH were associated with the stress-induced inhibition of ovarian progesterone production during early pregnancy. We conclude that chronic stress impacts endocrine networks differently in pregnant and nonpregnant mammals. These findings underscore the need for further studies exploring dynamic changes in endocrine networks participating in pregnancy initiation and progression to elucidate the physiological mechanisms that connect stress exposure to adverse pregnancy outcomes.

female reproduction; glucocorticoid; luteinizing hormone; ovary; prolactin

INTRODUCTION

Maternal stress increases the likelihood of adverse pregnancy outcomes in many mammals (31), including humans (10, 35). Adverse outcomes include total failure (miscarriage, or resorption) as well as a range of sublethal effects, including lower birth weight of offspring, slower growth rates, and altered social and anxiety behaviors (21, 22, 27). One mechanism by which stress can produce these adverse outcomes is by

increasing activity of the hypothalamic-pituitary-adrenal (HPA) axis; when animals experience stress, the HPA axis increases glucocorticoid release from the adrenal gland, and this release of glucocorticoids (above homeostatic levels) impacts pregnancy progression and fetal development (37, 38). Some of these effects result from the inhibition of the primary pregnancy maintenance hormone, progesterone. If progesterone is too low during early pregnancy, embryo implantation and/or the pregnancy will fail (8, 20, 33), and more broadly, low progesterone throughout pregnancy can adversely affect placental growth and development (7).

Circulating progesterone during early pregnancy in humans and other mammals is inversely correlated with circulating glucocorticoids (15, 19, 28), and stress exposure during pregnancy is associated with lower circulating progesterone concentrations (25, 28, 39). Despite clear evidence of these associations, the pathway by which glucocorticoids inhibit progesterone production during pregnancy is unknown (28, 37).

In nonpregnant female mammals, glucocorticoids regulate ovarian function primarily through action on the hypothalamus and pituitary. For example, in nonpregnant females, glucocorticoids alter hypothalamic and pituitary hormone release [including luteinizing hormone (LH) and prolactin (PRL)], and these changes can result in lower sex steroid production (estrogens and progestogens) from the ovary (37). The association between glucocorticoids and progesterone release during pregnancy could potentially reflect action through these same circuits. However, as described below, pregnancy requires substantial changes to regulatory networks and activity of endocrine axes (the reproductive axis being only one of many), and it is therefore possible that the association between glucocorticoid and progesterone production during pregnancy results from novel, as yet unidentified interactions among endocrine organs or from interactions that are less important in nonpregnant females. Furthermore, pregnancy is dynamic, and the effects of chronic stress on endocrine outcome measures (including glucocorticoid and progesterone production) are likely to change across pregnancy progression.

In rodents, the amount of progesterone produced during pregnancy depends on steroidogenic activity in the corpora lutea in the ovary. Increased steroidogenic activity by the corpora lutea is a function of activity across two pathways (26, 34). First, inhibition of the enzyme 20 α HSD, which usually metabolizes progesterone and second, increased expression of steroidogenic enzymes, especially P450 cholesterol side-chain cleavage enzyme (P450SCC). The pituitary hormones PRL and LH control these pathways, respectively (3, 4, 26, 34). Failure

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or decreased function of any of these signaling pathways within the ovary during early pregnancy can increase the likelihood of adverse pregnancy outcomes (3, 14, 17). Though the placenta begins to contribute progesterone to circulation by midpregnancy (32, 34), the ovary is thought to be required for pregnancy maintenance throughout gestation in mice (23).

We hypothesized that chronic stress affects ovarian steroidogenesis across the first half of pregnancy (early to midpregnancy) by modulating the pituitary hormones (LH, PRL) that mediate these responses in nonpregnant animals. To test this possibility, we used chronic restraint to model chronic stress in mice, and we measured pituitary and ovarian hormone production and gene expression in candidate ovarian steroidogenic pathways during early and midpregnancy. We predicted that restrained females would have elevated circulating concentrations of glucocorticoids, specifically corticosterone, which would be associated with lower circulating progesterone. Furthermore, we predicted that the pituitary hormone signaling (circulating concentrations of PRL or LH and receptor expression in the ovary) would be concomitantly lower in restrained females.

METHODS

Animals. C57BL/6J mice were purchased from the Jackson Laboratory (Sacramento, CA) and housed in ventilated cages on a 14:10 light/dark cycle (lights on at 0600, lights off at 2000) with ad libitum access to food and water. Experimental animals were pair-housed with the males throughout the experiment. All animals were allowed to acclimate for at least 1 wk. Females used in these experiments were 8–10 wk old. All protocols were approved by the University of California Berkeley Office of Laboratory Animal Care and were consistent with NIH guidelines for the care and use of laboratory animals.

Experimental procedures. Successful mating was determined either through observation of at least two ejaculations during timed mating trials or by the identification of a vaginal plug the morning following pairing. The morning after mating or on which a vaginal plug was found was considered *day 1* of pregnancy. Females were then pseudo-randomly assigned to restraint stress or control (unrestrained) groups such that females that mated on the same day were distributed across experimental groups. In this way, assignment between groups was balanced across the length of the experiment (see Table 1 for total sample sizes). All females were weighed each morning before treatment. Animals assigned to the chronic restraint stress group were moved each morning, beginning on *day 1*, to a separate room where they were restrained in a modified 50 ml plastic tube. Animals were also exposed to predator odor during restraint. Each day, 15 μ l of predator odor (undiluted fox urine, Minnesota Trapline, Inc., Pannock, MN) was freshly soaked into a new cotton ball and placed in the cage with each mouse during restraint. Daily restraint lasted 4 h from 0800 to 1200 (relative to lights on). Restraint was repeated daily until tissue collection. Unrestrained females remained in their home cages.

Females were euthanized on either *day 5* (early) or *day 10* (mid-) pregnancy. All animals were euthanized via intraperitoneal injection

of sodium pentobarbital (200 mg/kg) followed by rapid decapitation or perfusion. In animals euthanized via decapitation, trunk blood was collected into 1.5-ml Eppendorf tubes, and the ovaries were rapidly dissected from the body, cleaned of fat, and flash-frozen in isopentane on dry ice. The number of developing fetuses for each side of the uterus was counted in females collected at midpregnancy, and fetal developmental abnormalities or resorption sites were recorded by an observer unaware of the individual's treatment. In animals euthanized via perfusion, blood was collected via the retro-orbital sinus immediately before perfusion; the uterus and ovaries were clamped and removed before perfusion, and these tissues were immediately dissected and frozen as previously described. Tissues were stored at -80°C until extraction and analysis. Blood was centrifuged at 1,300 relative centrifugal force for 10 min and serum removed. Serum was centrifuged a second time for 1 min and then aliquoted and stored at -80°C . Blood samples were collected an average of 2:41 (SD 0:30) min from lifting the cage [average (SD)] with a median time to collection of 2:37 ($n = 46$). Samples collected more than 4 min after lifting the cage were excluded from analyses.

Hormone analyses. Progesterone was quantified using Cayman Chemical Progesterone ELISA (cat. no. 582601, Ann Arbor, MI). Intra- and interassay variations for progesterone were 3.9% and 5.1%, respectively. Baseline corticosterone was quantified using Enzo corticosterone ELISA kit (ADI-900–097; Enzo Life Sciences, Inc., Farmingdale, NY) using the manufacturer's protocol for small sample volumes. Intra- and interassay variations were 4.8% and 7.9%, respectively. LH levels were quantified using an LH ELISA, modified from (2). The protocol was kindly provided by Jens D. Mikkelsen (Copenhagen University Hospital, Denmark). Briefly, 96-well microtiter plates were coated with 50 μ l of bovine LH β 518B7 monoclonal antibody (kindly provided by Lillian E. Sibley, UC Davis) and incubated overnight at 4°C . Excess antibody was removed, and the plates were washed with 200 μ l/well of 10 mM PBS with 0.05% Tween 20. The plates were blocked using 5% skim milk powder in PBS with 0.05% Tween 20 and incubated for 1 h at room temperature. Following washes, 50 μ l of sample or standards of mouse LH [mouse radioimmunoassay (RIA) kit, AF Parlow, National Hormone and Pituitary program, University of California, Harbor Medical Center, Los Angeles, CA], diluted in assay buffer, were added per well in duplicates and incubated for 2 h at room temperature. The plates were washed, and 50 μ l of rabbit polyclonal LH antibody (AFP240580Rb, AF Parlow, National Hormone and Pituitary program, University of California, Harbor Medical Center, Los Angeles, CA) were added into each well, then incubated at room temperature for 90 min. After washing, 50 μ l polyclonal goat anti-rabbit IgG conjugated to horseradish peroxidase (cat. no. P0448, DAKO Cytomation) was added at 1:2,000 dilution and incubated for 1 h at room temperature. After washing, 100 μ l of o-Phenylenediamine (Invitrogen, cat. no. 00–2003) in citrate buffer were added to all the wells. The color reaction was allowed to develop for 30 min in the dark. The enzyme was stopped by adding 50 μ l of 3 M HCl per well and the optical density of each well was immediately read at 490 nm with a reference of 655 nm.

Samples that did not reach the limit for detection for the LH assay were assigned the lowest measurable value (0.078 ng/ml; $n = 7$, all females from midpregnancy). Intra- and interassay variations were 5.9% and 3.59%, respectively.

Three samples (all in the midpregnancy group) gave values that were nearly 10 times greater than the average of all other samples [1.19, 1.71, and 2.12 ng/ml compared with the average of 0.18 ng/ml (range: 0.078–0.53 ng/ml)]. Such values are comparable to LH values measured in ovariectomized mice that were run in the same assay as internal controls. However, we could not determine any reason to suspect that the values measured were inaccurate. Accordingly, we include these data points in the figure and present analyses with and without these samples included.

Table 1. Summary sample sizes used in experiment

Treatment	Day of Pregnancy Collected		Total
	Day 5	Day 10	
Control	10	12	22
Restrained	9	15	24
		Total	46

Table 2. Primers used for quantitative PCR analyses of gene expression in C57BL/6J mice

Target	Forward Primer	Reverse Primer	T _A , °C
TBP	GGGAGAATCATGGACCAG	GGCTGTGGAGTAAGTCCTGT	55
PRLRL	ATAAAAGGATTTGATACTCATCTGCTAGAG	TGTCATCCACTTCCAAGAAGTCC	60
StAR	CTTGGCTGCTCAGTATTGAC	TGGTGGACAGTCCTTAACAC	55
P450SCC	CGATACTCTTCTCATGCGAG	CTTTCTTCCAGGCATCTGAAC	55
LHR	CTCCAGAGTTGT CAGGGTCCG	AGGTGAGAGATAGTCGGGCG	60
20 α HSD	ATGAGCTTTTGCCTAAAGATGAG	GTTAGACACCCCGATGGAC	55

Primers for PRLRL were taken from (6). LHR, luteinizing hormone receptor; PRLRL, long-form prolactin receptor; StAR, steroidogenic acute regulatory protein; T_A, annealing temperature.

PRL was assayed using the mouse prolactin ELISA kit from Abcam (cat. no. ab-100736, Cambridge, MA). Intra-assay variation for PRL was 2.8%.

Some samples did not have sufficient serum to quantify all hormones, thus sample sizes vary for different hormone measures.

Gene expression analysis. Total RNA was extracted from whole ovaries (ISOLATE II RNA Mini-kit, BIO-52073, Bionline USA Inc., Taunton, MA). The RNA quality of a random subset of samples ($n = 10$) was analyzed on an Agilent Technologies Bioanalyzer and yielded an average RNA integrity number of 9.5 (range: 8.8 to 10). We reverse transcribed 1.0 μ g of RNA (iScript Advanced cDNA synthesis Kit for RT-qPCR, Bio-Rad Laboratories Inc., Hercules, CA). cDNA was diluted 1:25 in nuclease-free water immediately before performing quantitative PCR. Quantitative PCR was performed using duplicate 10- μ l reactions with a 2-step amplification for 40 cycles followed by a melt curve. All primers used were validated before analyses by confirming single-peak melt curves, correct product length, and acceptable efficiency (all primer pairs between 85% and 101% efficiency). Primer sequences and annealing temperature are provided in Table 2. Any wells with aberrant melt-curves were excluded from expression analysis. C_T values were corrected for efficiency, and relative expression was calculated using methods by Pfaffl and colleagues (29). All data are expressed as fold-change over midpregnancy (day 10), restrained individuals.

Statistical analyses. All analyses were run in RStudio 0.98.1091 with the nlme and multcomp packages.

We evaluated the change in mass across pregnancy in the restrained and unrestrained groups by calculating percent change in mass per day (of initial body mass) from days 1–6 and days 6–9. We identified day 6 as the point at which chronically stressed females began to gain mass by visually inspecting mass across pregnancy (Fig. 1). Slope was statistically evaluated using a one-way ANOVA (hereafter, AOV) with post hoc comparisons among means using a Holms-Sidak correction for multiple comparisons.

Progesterone, corticosterone, and LH were log-transformed for analyses to meet assumptions of residual normality. Blood collection method (retro-orbital vs. trunk blood) significantly affected progesterone measurements, and because samples collected from animals at day 5 were all collected via retro-orbital bleeds, we only included progesterone measurements from day 10 animals that were collected via the retro-orbital sinus in the analysis. We ran a one-way ANOVA with planned contrasts to test for differences among groups based on our a priori predictions. We tested for differences between restrained and unrestrained individuals during early and midpregnancy (planned contrasts 1 and 2), and we tested for differences between early and midpregnancy (planned contrast 3). The correlations between baseline corticosterone and progesterone were assessed using Pearson's product-moment correlation. A difference in circulating PRL between restrained and unrestrained animals during early pregnancy was analyzed using Welch's two sample *t*-test.

Gene expression analyses were carried out using a repeated-measures linear regression model including a random effect of individual to account for use of both ovaries. All genes were log-transformed to fulfill assumptions of normality of residuals. Again, we used planned contrasts to test for a priori differences. We used Pearson's product-moment correlation to examine correlations between expressed genes. All tests were considered statistically significant at $P < 0.05$. Because we used planned comparisons, we did not correct *P* values for multiple comparisons. Figures show untransformed data and use mean \pm SE, except where noted.

RESULTS

Females exposed to chronic restraint stress lost body mass during early pregnancy in contrast to unrestrained (CON) females, which gained mass (Fig. 1A). Once chronically stressed (STR) females began gaining mass (after day 6), they gained mass at the same rate as unrestrained animals (one-way

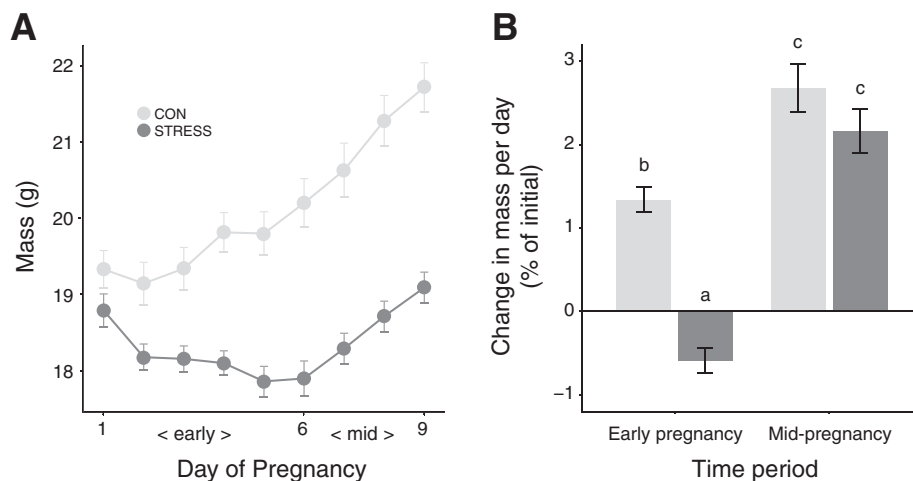


Fig. 1. A: patterns of weight gain across pregnancy in unrestrained mice (CON, light line; $n = 20$) and mice exposed to chronic restraint stress (STRESS, dark line; $n = 23$). B: when exposed to chronic restraint stress, pregnant female mice (dark bar) lost mass during early pregnancy (days 1 to 6) relative to pregnant unrestrained mice, which gained weight (light bar). However, during mid-pregnancy (after day 6), chronically stressed females gained mass at rates comparable to unrestrained females (though absolute mass is still less than unrestrained females; see 2A). ^{a,b,c}Significantly different post hoc comparison ($P < 0.03$ for all).

ANOVA: $F_{3,42} = 44.07$, $P < 5e^{-13}$; Fig. 1B). Regardless of treatment, pregnant mice gained mass between successful mating and midpregnancy (CON: 2.80 ± 0.13 g; STR: 0.63 ± 0.12 g); however, unrestrained females gained more mass (Welch's t -test, $t_{17,5} = 12.03$, $P < 7e^{-10}$; Fig. 2A).

There were no overall differences in total number of fetuses per female at midpregnancy (data not shown, $P > 0.3$). However, evidence of early resorption and underdeveloped fetuses was apparent in 2 of 15 females (13%) exposed to daily restraint stress, whereas 0 of 12 (0%) unrestrained females showed signs of resorption or underdeveloped fetuses.

Steroid hormones. Baseline corticosterone (CORT) increased as pregnancy progressed (Fig. 2A; AOV: $F_{3,28} = 10.65$, $P < 8e^{-5}$; Pregnancy: $t = 2.573$, $P < 0.015$). Chronic restraint stress elevated baseline CORT during both early and midpregnancy (Fig. 2A; AOV: $F_{3,28} = 10.65$, $P < 8e^{-5}$; planned contrasts: early: $t = 2.554$, $P < 0.016$; mid: $t = 4.11$, $P < 0.0003$). Chronic stress also resulted in lower circulating progesterone but only during early pregnancy (Fig. 2B; AOV: $F_{3,26} = 16.26$, $P < 4e^{-6}$; planned contrasts: early: $t = -5.776$, $P < 5e^{-6}$; mid: $t = 0.492$, $P < 0.63$). Baseline CORT was correlated with circulating progesterone during early pregnancy (Fig. 2C; Pearson $R = -0.78$, $t_7 = -3.32$, $P < 0.013$) but not during midpregnancy (Pearson $R = 0.16$, $t_9 = 0.48$, $P > 0.60$).

Pituitary hormones. Circulating PRL did not differ between unrestrained and chronically restrained females during early pregnancy (Fig. 3A; Welch's t -test $t_{7,984} = -1.27$, $P = 0.24$). When all LH measures are included the analyses, circulating LH did not vary across pregnancy or with treatment ($P > 0.15$ for all). However, these three points in the midpregnancy group (see Fig. 3B) are all at least two times greater than any other measured value and when included they are responsible for a fivefold increase in standard deviation within the midpregnancy group. When these points are excluded, circulating LH was lower during midpregnancy relative to early pregnancy

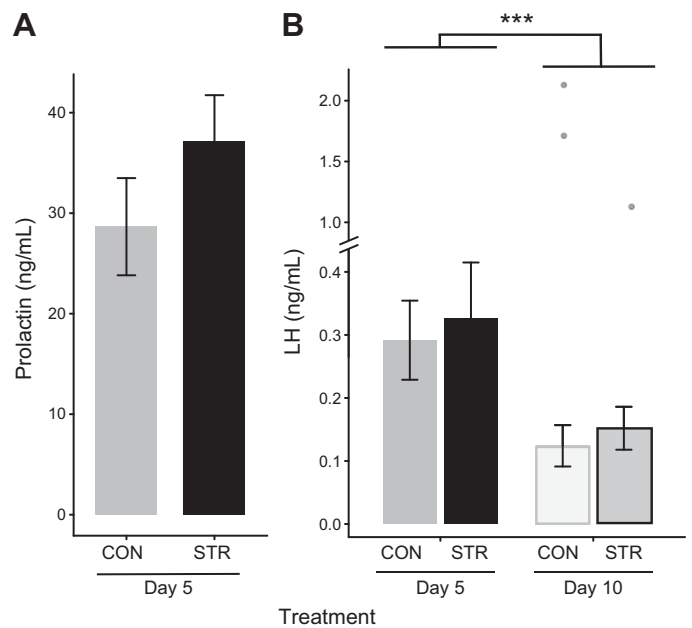


Fig. 3. A: circulating concentration of prolactin (PRL) did not differ between unrestrained (CON) and restraint-stressed (STR) pregnant mice on day 5 of pregnancy ($P > 0.2$). B: circulating concentration of luteinizing hormone (LH) varied between early and midpregnancy but not with stress. PRL day 5: CON, $n = 5$; STR, $n = 5$. LH day 5: CON, $n = 5$; STR, $n = 4$; day 10: CON, $n = 9$; STR, $n = 12$. *** $P < 0.001$, planned comparisons.

(AOV: $F_{3,26} = 4.711$, $P < 0.009$; planned contrasts: pregnancy: $t = -3.72$, $P < 0.0009$), though it still did not differ between unrestrained and chronically restrained females (Fig. 3B; planned contrasts: early: $t = 0.091$, $P < 0.93$; mid: $t = 0.773$, $P < 0.45$).

Ovarian gene expression. During early pregnancy, the expression of two steroidogenic enzymes [steroidogenic acute

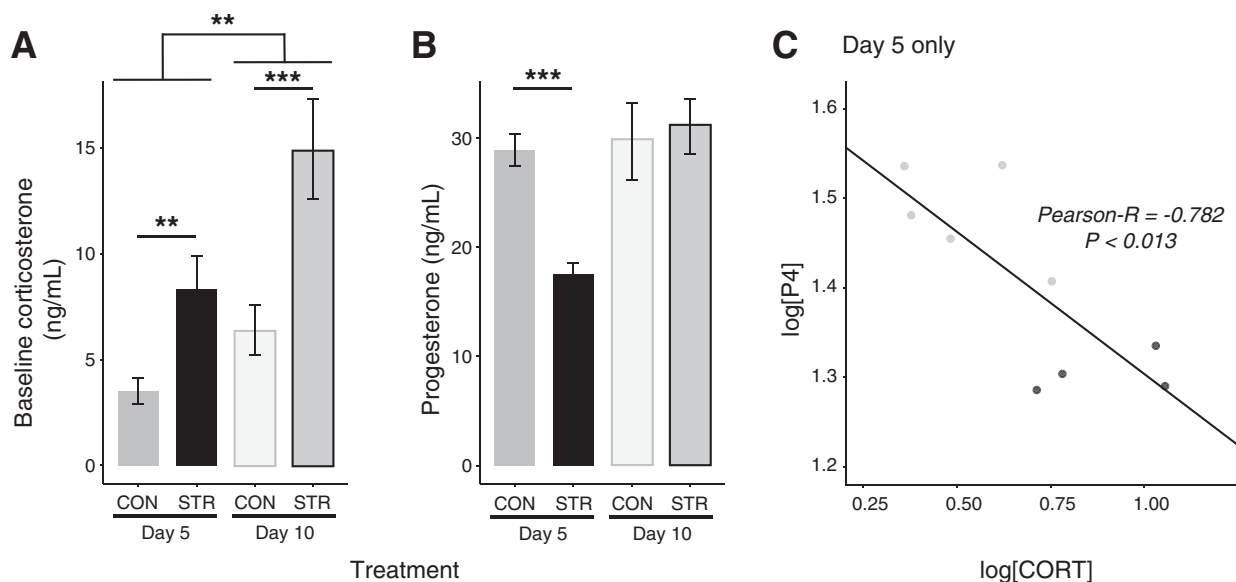


Fig. 2. A: baseline corticosterone was elevated in chronically stressed (STR), pregnant female mice relative to unrestrained (CON) females at both day 5 and day 10 of pregnancy. Baseline corticosterone (CORT) increased from early to midpregnancy (day 5 to day 10) and was elevated in restrained females relative to unrestrained at both time points. Day 5: CON, $n = 5$; STR, $n = 4$; day 10: CON, $n = 11$; STR, $n = 12$. B: baseline progesterone was lower in chronically stressed, pregnant female mice on day 5 relative to CON females but not on day 10. C: on day 5, baseline corticosterone was inversely correlated with circulating progesterone. Day 5: CON, $n = 10$; STR, $n = 9$; day 10: CON, $n = 5$; STR, $n = 6$. ** $P < 0.02$; *** $P < 0.001$, planned comparisons.

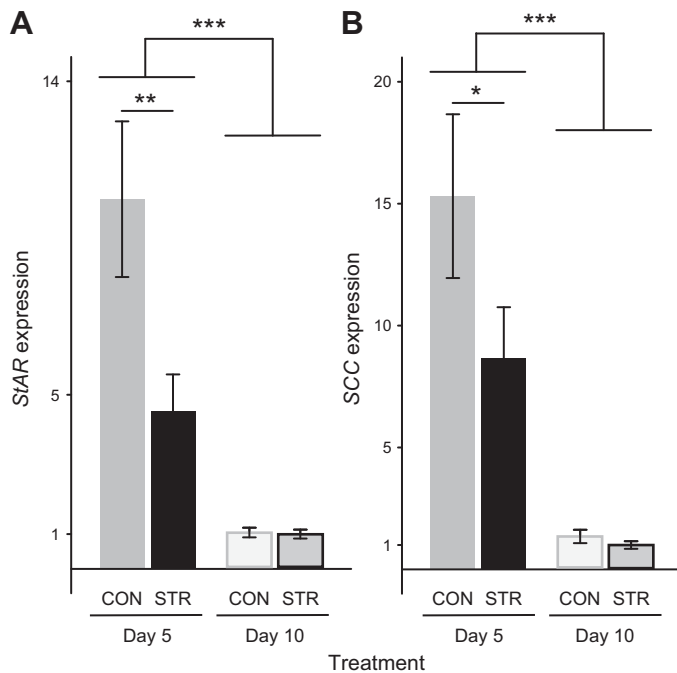


Fig. 4. Expression of two steroidogenic enzymes in the ovary is modulated by stress and pregnancy progression. **A:** fold-change expression of steroidogenic acute regulatory protein (*StAR*) was decreased in restraint-stressed (STR) animals during early pregnancy related to unrestrained females (CON) but downregulated in both groups during midpregnancy. **B:** fold-change expression of cholesterol side chain cleavage enzyme (*SCC*) was also lower in STR during early pregnancy but downregulated in both stressed and unrestrained animals during midpregnancy. Plot shows untransformed data. *Day 5:* CON, $n = 20$; STR, $n = 16$; *day 10:* CON, $n = 18$; STR, $n = 17$. Samples sizes report number of ovaries. Both ovaries were used from most individuals within a repeated measures analysis (see METHODS, *Statistical analyses*). * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$, planned comparisons.

regulatory protein (*StAR*) and P450 cholesterol side-chain cleavage enzyme (*SCC*) were lower in chronically stressed animals compared with unrestrained females (*StAR*, early: $t_{34,32} = -3.46$, $P < 0.0015$; *SCC*, early: $t_{34,32} = -2.41$, $P < 0.0220$; Fig. 4). Expression of these genes in the ovary during midpregnancy was lower relative to early pregnancy, and there was no difference in expression between chronically stressed and unrestrained individuals during midpregnancy (*StAR*, pregnancy: $t_{34,32} = -7.21$, $P < 0.0001$, mid: $t_{34,32} = -0.043$, $P < 0.96$; *SCC*, pregnancy: $t_{34,32} = -8.30$, $P < 0.0001$, mid: $t_{34,32} = -0.414$, $P < 0.68$; Fig. 4).

Expression of the long prolactin receptor isoform (*PRLRL*) was lower in restrained females during early pregnancy (early: $t = -2.28$, $P < 0.029$; Fig. 5A) but not during midpregnancy (mid: $t = -0.084$, $P < 0.93$), and there was no overall difference in expression between early and midpregnancy (pregnancy: $t = -0.772$, $P < 0.45$). Expression of the receptor for LH (*LHR*) and the enzyme *20 α HSD* decreased during midpregnancy relative to early pregnancy (*LHR*, pregnancy: $t = -4.62$, $P < 0.0001$; *20 α* , pregnancy: $t = -5.22$, $P < 0.0001$), but there was no difference related to stress treatment (*LHR*: early: $t = -0.59$, $P < 0.56$; mid: $t = 0.43$, $P < 0.67$; *20 α* : early: $t = -0.077$, $P < 0.94$; mid: $t = 0.62$, $P < 0.54$; Fig. 5, B and C).

We found a strong correlation between the expression of *PRLRL* and expression of the two steroidogenic enzymes (*StAR* and *SCC*; Fig. 6). The relationships between the steroidogenic

enzymes and *PRLRL* were consistent between early pregnancy (*day 5*; *SCC*: Pearson $R = 0.98$, $t_{34} = 29.60$, $P < 2.2e^{-16}$; *StAR*: Pearson $R = 0.97$, $t_{34} = 26.82$, $P < 2.2e^{-16}$), and midpregnancy (*Day 10*; *SCC*: Pearson $R = 0.83$, $t_{32} = 8.37$, $P < 1.47e^{-9}$; *StAR*: Pearson $R = 0.89$, $t_{32} = 11.25$, $P < 1.18e^{-12}$). The relationships between these gene transcripts shift in late pregnancy; the steroidogenic enzymes are downregulated, whereas there is no longer a difference between restrained and unrestrained females in *PRLRL* (see Fig. 4, A and B and Fig. 5A). However, the slope of the line that explains the correlation between steroidogenic enzymes and *PRLRL* appears to be similar (Fig. 6, A and B).

DISCUSSION

We found that ovarian progesterone production is sensitive to restraint stress. Our results suggest that glucocorticoids do not inhibit progesterone release during pregnancy via a top-down (hypothalamic-pituitary) mechanism within the HPG axis, because basal pituitary LH and PRL secretion were unaffected by stress. The downregulated expression of steroidogenic enzymes in the ovary by midpregnancy suggests that the majority of circulating progesterone at this time point may no longer be from the ovary. In contrast to the ovary during early pregnancy, midpregnancy progesterone synthesis appears resilient to restraint stress and elevated baseline glucocorticoids. Though there have been concerted efforts to understand the extent to which chronic stress alters reproductive outcomes, our results make it clear that there is substantial work still needed to describe and to test the basic interactions between the HPA and reproductive axes across different stages of pregnancy. Understanding the functional network between these and other endocrine axes during pregnancy will help to establish the mechanisms connecting maternal stress to reproductive failure.

Effects of stress-induced CORT release on progesterone during early pregnancy. The inverse correlation we found between baseline CORT and progesterone production during early pregnancy is consistent with other rodent studies (18, 19). Direct action of CORT on ovarian progesterone synthesis is unlikely to explain the relationship between circulating CORT and progesterone (for more, see 24, 26, 36, 37), and we found no evidence to support the hypothesis that chronic stress alters the basal release of pituitary hormones (LH and PRL) during early pregnancy. Instead, placental factors that regulate CORT metabolism (e.g., *11 β HSD*) and/or ovarian progesterone synthesis (e.g., placental lactogens) are promising areas for further study. Careful attention to placental endocrine activity and sensitivity in vivo during early pregnancy, especially related to glucocorticoid receptor isoform expression (5), may facilitate the identification of new functional mechanisms by which CORT impacts progesterone synthesis.

In addition, progesterone production and CORT secretion during pregnancy could be connected through other shared upstream regulators. Because restraint stress resulted in initial loss of body mass, suggesting that restrained females entered a negative energy balance, endocrine or metabolic signals associated with changes in energy balance could be responsible for changes to baseline CORT and progesterone production. For example, the adipose hormone leptin promotes ovarian progesterone production (13) is inhibited by chronic stress (11, 12) and is inversely related to CORT during negative energy balance in mice (1). Mapping the interactions between energy

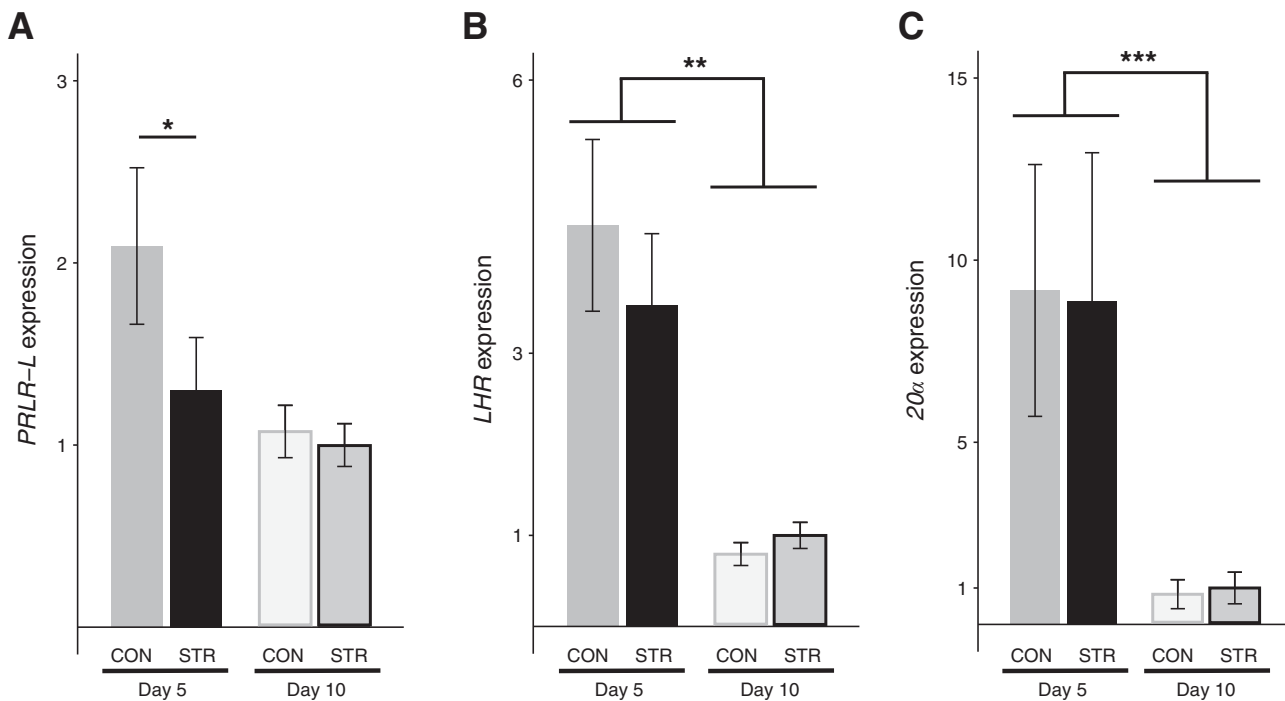


Fig. 5. Expression of other candidate genes known to be important for ovarian progesterone production during early pregnancy are unaffected by restraint stress. *A*: long-form prolactin receptor (*PRLRL*) was not inhibited by chronic restraint during early pregnancy but not during midpregnancy. *B*: luteinizing hormone receptor (*LHR*) was not affected by restraint stress during early pregnancy but showed substantial downregulation by midpregnancy in both groups. *C*: *20αHSD* (*20α*) was also not affected by restraint stress during early pregnancy but was downregulated in the ovary by midpregnancy in both groups. Plot shows untransformed data. Day 5: CON, $n = 20$; STR, $n = 16$; day 10: CON, $n = 18$; STR, $n = 17$. Samples sizes report number of ovaries. Both ovaries were used from most individuals within a repeated measures analysis (see METHODS, *Statistical analyses*). * $P < 0.05$; ** $P < 0.02$; *** $P < 0.001$, planned comparisons.

balance circuits and reproductive function specifically in early pregnancy is likely to identify new connections between stress and adverse pregnancy outcomes.

Progesterone production during midpregnancy. In midpregnancy, *StAR* and *SCC* were dramatically downregulated relative to early pregnancy, and there were no longer any differences in gene expression between restrained and unrestrained females. The decrease in expression of *StAR* and *SCC* suggests that ovarian steroidogenic activity is lower in midpregnancy. Although these results counter the classic suggestion that the ovary is required for progesterone production throughout pregnancy in mice (23), they are consistent with the idea that decreasing pituitary LH release by midpregnancy causes a decline in ovarian progesterone production (34). In further support of the latter idea, circulating LH concentrations were lower during midpregnancy relative to early pregnancy in this study. We also found a novel correlation between *PRLRL* and *StAR* and *SCC*. The strong coregulation between these genes and differential sensitivity to stress across pregnancy underscores the need to understand the regulatory networks that control ovarian progesterone production better.

Even though restrained females continued to exhibit elevated baseline CORT during midpregnancy, circulating progesterone no longer differed between restrained and unrestrained females. Moreover, even though progesterone remained elevated, steroidogenic genes in the ovary were considerably downregulated, suggesting the ovary is much less steroidogenically active by midpregnancy. Circulating progesterone during midpregnancy may instead reflect placental steroidogenesis. Interestingly, circulating progesterone appears to

be insensitive to chronic stress (elevated CORT) during midpregnancy. Further work to establish the source of midpregnancy progesterone is needed to determine how the apparent insensitivity to CORT develops across pregnancy.

Caveats. Though our results present a relatively clear picture of how chronic stress affects reproductive function during the first half of pregnancy, there are some important caveats. First, only ovarian mRNA, not protein, was measured, raising the possibility that protein expression and activity may differ meaningfully. Second, both PRL and LH are released in a pulsatile fashion such that single time point measurement may miss dynamic changes in the pulse rate or peak size for either hormone resulting from chronic stress exposure. The three LH samples showing exceptionally high values may reflect the pulsatile nature of this hormone, whereas the majority (31/33) measures reflect basal levels as expected. Evaluating upstream changes in protein and gene expression within the pituitary and hypothalamus and/or serial blood samples would be required to determine conclusively whether temporal changes in pituitary hormone production and release could explain the relationship between glucocorticoids and progesterone during early pregnancy. However, most studies evaluating the effects of stress on pituitary hormone release (LH in particular) find differences using single time point measures (e.g., 16, 30), and we were able to detect a change in LH across pregnancy.

More broadly, it is worth considering that animals or people that experience chronic stress during pregnancy are likely to experience stress before pregnancy as well. Geraghty et al. (9) showed that chronic stress before pregnancy in rats was associated with lower reproductive success, but that these effects could be

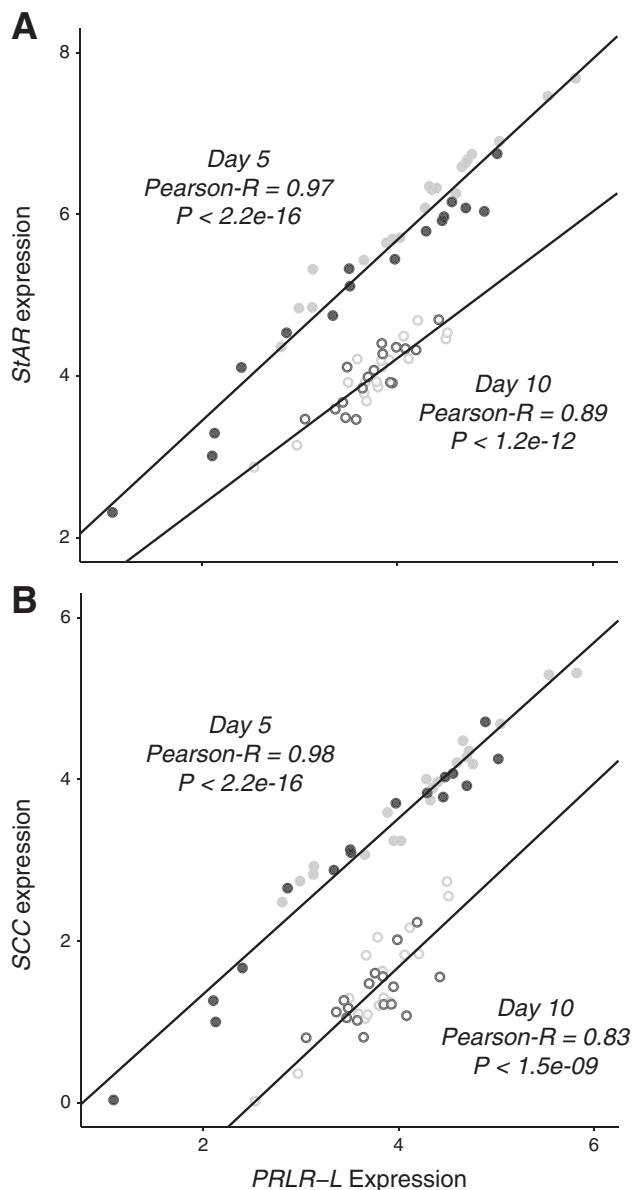


Fig. 6. Expression of the long-form of the prolactin receptor (*PRLRL*) was correlated with expression of steroidogenic enzymes in the ovaries (light gray circles: day 5, unrestrained; dark gray circles: day 5, restraint stress; dark gray open circles: day 10, unrestrained; light gray open circles: day 10, restrained). A: *PRLRL* covaries with expression of steroidogenic acute regulatory protein (*StAR*) across pregnancy. B: *PRLRL* covaries with expression of side chain cleavage enzyme (*SCC*) across pregnancy. Plot shows log-transformed data.

ameliorated by inhibiting production of a stress-induced neuropeptide, gonadotropin-inhibitory hormone, in the hypothalamus leading up to pregnancy. Thus, their results demonstrate that effects of chronic stress on central (hypothalamic) processes can explain some stress-related reproductive failures that occur during pregnancy. Whether we can differentiate between mechanisms that come into play before pregnancy versus during pregnancy and furthermore, whether this difference is functionally meaningful will be important moving forward.

Conclusions. Taken together, our results present a first step toward identifying the endocrine network that connects psychological stress to reproductive function during early preg-

nancy. Importantly, the effects of chronic stress on the reproductive axis during pregnancy do not seem to be acting through the well-known circuits that play a role in nonpregnant females. Combining hormone production measures and ovarian gene expression across pregnancy progression offers a new perspective for understanding the endocrine networks through which stress impacts pregnancy.

ACKNOWLEDGMENTS

We acknowledge Aimee Pepper, Pooja Srinivas, Amber Kirawala, Laura Reynolds, Damhee Hu, Emily Tang, Alvin Balmeo, and especially Veronica Kim, whose help made this project possible.

GRANTS

This material is based upon work supported in part by the National Science Foundation Graduate Research Fellowship to K. Wilsterman (Grant No. DGE-106400) and an NIH Grant to L. Kriegsfeld (Grant No. HD-050470).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.W., N.G., L.J.K., and G.E.B. conceived and designed research; K.W. and N.G. performed experiments; K.W. analyzed data; K.W., N.G., L.J.K., and G.E.B. interpreted results of experiments; K.W. prepared figures; K.W. drafted manuscript; K.W., N.G., L.J.K., and G.E.B. edited and revised manuscript; K.W., N.G., L.J.K., and G.E.B. approved final version of manuscript.

REFERENCES

- Ahima RS, Prabakaran D, Mantzoros C, Qu D, Lowell B, Maratos-Flier E, Flier JS. Role of leptin in the neuroendocrine response to fasting. *Nature* 382: 250–252, 1996. doi:10.1038/382250a0.
- Ancel C, Bentsen AH, Sébert M-E, Tena-Sempere M, Mikkelsen JD, Simonneaux V. Stimulatory effect of RFRP-3 on the gonadotrophic axis in the male Syrian hamster: the exception proves the rule. *Endocrinology* 153: 1352–1363, 2012. doi:10.1210/en.2011-1622.
- Bachelot A, Beaufaron J, Servel N, Kedzia C, Monget P, Kelly PA, Gibori G, Binart N. Prolactin independent rescue of mouse corpus luteum life span: identification of prolactin and luteinizing hormone target genes. *Am J Physiol Endocrinol Metab* 297: E676–E684, 2009. doi:10.1152/ajpendo.91020.2008.
- Binart N, Helloc C, Ormandy CJ, Barra J, Clément-Lacroix P, Baran N, Kelly PA. Rescue of preimplantary egg development and embryo implantation in prolactin receptor-deficient mice after progesterone administration. *Endocrinology* 141: 2691–2697, 2000. doi:10.1210/endo.141.7.7568.
- Clifton VL, Cuffe J, Moritz KM, Cole TJ, Fuller PJ, Lu NZ, Kumar S, Chong S, Saif Z. Review: The role of multiple placental glucocorticoid receptor isoforms in adapting to the maternal environment and regulating fetal growth. *Placenta* 54: 24–29, 2017. doi:10.1016/j.placenta.2016.12.017.
- Corbacho AM, Valacchi G, Kubala L, Olano-Martín E, Schock BC, Kenny TP, Cross CE. Tissue-specific gene expression of prolactin receptor in the acute-phase response induced by lipopolysaccharides. *Am J Physiol Endocrinol Metab* 287: E750–E757, 2004. doi:10.1152/ajpendo.00522.2003.
- Cross JC, Werb Z, Fisher SJ. Implantation and the placenta: key pieces of the development puzzle. *Science* 266: 1508–1518, 1994. doi:10.1126/science.7985020.
- Csapo AI, Wiest WG. An examination of the quantitative relationship between progesterone and the maintenance of pregnancy. *Endocrinology* 85: 735–746, 1969. doi:10.1210/endo-85-4-735.
- Geraghty AC, Muroy SE, Zhao S, Bentley GE, Kriegsfeld LJ, Kaufner D. Knockdown of hypothalamic RFRP3 prevents chronic stress-induced infertility and embryo resorption. *eLife* 4: e04316, 2015. doi:10.7554/eLife.04316.
- Glover V. Maternal depression, anxiety and stress during pregnancy and child outcome; what needs to be done. *Best Pract Res Clin Obstet Gynaecol* 28: 25–35, 2014. doi:10.1016/j.bpobgyn.2013.08.017.
- Harris RBS. Chronic and acute effects of stress on energy balance: are there appropriate animal models? *Am J Physiol Regul Integr Comp Physiol* 308: R250–R265, 2015. doi:10.1152/ajpregu.00361.2014.
- Harris RBS, Mitchell TD, Simpson J, Redmann SM Jr, Youngblood BD, Ryan DH. Weight loss in rats exposed to repeated acute restraint

- stress is independent of energy or leptin status. *Am J Physiol Regul Integr Comp Physiol* 282: R77–R88, 2002. doi:10.1152/ajpregu.2002.282.1.R77.
13. Hausman GJ, Barb CR, Lents CA. Leptin and reproductive function. *Biochimie* 94: 2075–2081, 2012. doi:10.1016/j.biochi.2012.02.022.
 14. Ioannidis G, Sacks G, Reddy N, Seyani L, Margara R, Lavery S, Trew G. Day 14 maternal serum progesterone levels predict pregnancy outcome in IVF/ICSI treatment cycles: a prospective study. *Hum Reprod* 20: 741–746, 2005. doi:10.1093/humrep/deh644.
 15. Kajaysri J, Nokkaew W. Assessment of pregnancy status of Asian elephants (*Elephas maximus*) by measurement of progestagen and glucocorticoid and their metabolite concentrations in serum and feces, using enzyme immunoassay (EIA). *J Vet Med Sci* 76: 363–368, 2014. doi:10.1292/jvms.13-0103.
 16. Kirby ED, Geraghty AC, Ubuka T, Bentley GE, Kaufer D. Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *Proc Natl Acad Sci USA* 106: 11324–11329, 2009. doi:10.1073/pnas.0901176106.
 17. Larson SF, Butler WR, Currie WB. Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. *J Dairy Sci* 80: 1288–1295, 1997. doi:10.3168/jds.S0022-0302(97)76058-2.
 18. Liu G, Dong Y, Wang Z, Cao J, Chen Y. Restraint stress alters immune parameters and induces oxidative stress in the mouse uterus during embryo implantation. *Stress* 17: 494–503, 2014. doi:10.3109/10253890.2014.966263.
 19. Michel CL, Bonnet X. Effect of a brief stress on progesterone plasma levels in pregnant and non-pregnant guinea pigs. *Anim Biol* 64: 19–29, 2014. doi:10.1163/15707563-00002428.
 20. Milligan SR, Finn CA. Minimal progesterone support required for the maintenance of pregnancy in mice. *Hum Reprod* 12: 602–607, 1997. doi:10.1093/humrep/12.3.602.
 21. Mueller BR, Bale TL. Early prenatal stress impact on coping strategies and learning performance is sex dependent. *Physiol Behav* 91: 55–65, 2007. doi:10.1016/j.physbeh.2007.01.017.
 22. Mueller BR, Bale TL. Sex-specific programming of offspring emotionality after stress early in pregnancy. *J Neurosci* 28: 9055–9065, 2008. doi:10.1523/JNEUROSCI.1424-08.2008.
 23. Murr SM, Stabenfeldt GH, Bradford GE, Geschwind II. Plasma progesterone during pregnancy in the mouse. *Endocrinology* 94: 1209–1211, 1974. doi:10.1210/endo-94-4-1209.
 24. Myers M, Lamont MC, van den Driesche S, Mary N, Thong KJ, Hillier SG, Duncan WC. Role of luteal glucocorticoid metabolism during maternal recognition of pregnancy in women. *Endocrinology* 148: 5769–5779, 2007. doi:10.1210/en.2007-0742.
 25. van Niekerk CH, Morgenthal JC. Fetal loss and the effect of stress on plasma progestagen levels in pregnant Thoroughbred mares. *J Reprod Fertil Suppl* 32: 453–457, 1982.
 26. Niswender GD, Juengel JL, Silva PJ, Rollyson MK, McIntush EW. Mechanisms controlling the function and life span of the corpus luteum. *Physiol Rev* 80: 1–29, 2000. doi:10.1152/physrev.2000.80.1.1.
 27. Pankevich DE, Mueller BR, Brockel B, Bale TL. Prenatal stress programming of offspring feeding behavior and energy balance begins early in pregnancy. *Physiol Behav* 98: 94–102, 2009. doi:10.1016/j.physbeh.2009.04.015.
 28. Parker VJ, Douglas AJ. Stress in early pregnancy: maternal neuroendocrine-immune responses and effects. *J Reprod Immunol* 85: 86–92, 2010. doi:10.1016/j.jri.2009.10.011.
 29. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res* 29: e45, 2001. doi:10.1093/nar/29.9.e45.
 30. Pierce BN, Hensworth PH, Rivalland ETA, Wagenmaker ER, Morrissey AD, Papargiris MM, Clarke IJ, Karsch FJ, Turner AI, Tilbrook AJ. Psychosocial stress suppresses attractiveness, proceptivity and pulsatile LH secretion in the ewe. *Horm Behav* 54: 424–434, 2008. doi:10.1016/j.yhbeh.2008.04.005.
 31. Sheriff MJ, Bell A, Boonstra R, Dantzer B, Lavergne SG, McGhee KE, MacLeod KJ, Winandy L, Zimmer C, Love OP. Integrating ecological and evolutionary context in the study of maternal stress. *Integr Comp Biol* 57: 437–449, 2017. doi:10.1093/icb/ix105.
 32. Soares MJ, Talamantes F. Gestational effects on placental and serum androgen, progesterone and prolactin-like activity in the mouse. *J Endocrinol* 95: 29–36, 1982. doi:10.1677/joe.0.0950029.
 33. Spencer TE, Bazer FW. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front Biosci* 7: d1879–d1898, 2002. doi:10.2741/spencer.
 34. Stocco C, Telleria C, Gibori G. The molecular control of corpus luteum formation, function, and regression. *Endocr Rev* 28: 117–149, 2007. doi:10.1210/er.2006-0022.
 35. Van den Bergh BRH, van den Heuvel MI, Lahti M, Braeken M, de Rooij SR, Entringer S, Hoyer D, Roseboom T, Räikkönen K, King S, Schwab M. Prenatal developmental origins of behavior and mental health: The influence of maternal stress in pregnancy. *Neurosci Biobehav Rev* S0149-7634(16)30734-5, 2017. doi:10.1016/j.neubiorev.2017.07.003.
 36. Whirledge S, Cidlowski JA. Glucocorticoids, stress, and fertility. *Minerva Endocrinol* 35: 109–125, 2010.
 37. Whirledge S, Cidlowski JA. A role for glucocorticoids in stress-impaired reproduction: beyond the hypothalamus and pituitary. *Endocrinology* 154: 4450–4468, 2013. doi:10.1210/en.2013-1652.
 38. Whirledge S, Cidlowski JA. Glucocorticoids and reproduction: traffic control on the road to reproduction. *Trends Endocrinol Metab* 28: 399–415, 2017. doi:10.1016/j.tem.2017.02.005.
 39. Wiebold JL, Stanfield PH, Becker WC, Hillers JK. The effect of restraint stress in early pregnancy in mice. *J Reprod Fertil* 78: 185–192, 1986. doi:10.1530/jrf.0.0780185.