

Variation in maternal urinary cortisol profiles across the peri-conceptual period: a longitudinal description and evaluation of potential functions

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STUDY QUESTION: How do women's first morning urinary cortisol levels, a marker of stress axis activity, vary during the peri-conceptual period (the 12 weeks around conception)?

SUMMARY ANSWER: First morning urinary cortisol follows an overall increasing trajectory across the peri-conceptual period, interrupted by 2 week-long decreases during the week preceding conception and the fifth week following conception.

WHAT IS KNOWN ALREADY: Later gestational stages (i.e. second and third trimesters) are characterized by increasing levels of circulating cortisol. This increase is hypothesized to constitute a response to the energy demands imposed by fetal growth, and the development of energy reserves in preparation for nursing and performing regular activities while carrying pregnancy's extra weight and volume.

STUDY DESIGN, SIZE, DURATION: This study is based on a data set collected as part of a longitudinal, naturalistic investigation into the interactions between the stress (hypothalamic-pituitary-adrenal axis (HPAA)) and reproductive (hypothalamic-pituitary-gonadal axis (HPGA)) axes. Biomarkers of HPAA and HPGA function were quantified in first morning urinary specimens collected every other day from 22 healthy women who conceived a pregnancy during the study. We analyzed the longitudinal within- and between-individual variation in first morning urinary cortisol levels across the 12-week peri-conceptual period.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Participants were recruited from two rural, aboriginal, neighboring communities in Guatemala. Cortisol, estradiol and progesterone metabolites (estrone-3-glucuronide and pregnenediol glucuronide, respectively) and hCG levels were quantified in first morning urinary specimens using immunoassays to determine time of conception and confirm pregnancy maintenance. Linear mixed-effects models with regression splines were used to evaluate the magnitude and significance of changes in cortisol trajectories.

MAIN RESULTS AND THE ROLE OF CHANCE: Overall, maternal first morning urinary cortisol increased from 6 weeks prior to conception (geometric mean \pm SD = 58.14 \pm 36.00 ng/ml) to 6 weeks post-conception (89.29 \pm 46.76 ng/ml). The magnitude of the increase between the pre- and post-conception periods varied significantly between women (likelihood ratio test statistic = 8.0017, P = 0.005). The peri-conceptual period is characterized by an increasing cortisol trajectory (+1.36% per day; P = 0.007) interrupted by a week-long decline immediately prior to conception (-4.02% per day; P = 0.0013). After conception cortisol increased again (+1.73% per day; P = 0.0008) for 4 weeks, fell in the fifth week (-6.60% per day; P = 0.0002) and increased again in post-conceptual week 6 (+8.86% per day; P = 0.002). Maternal urinary cortisol levels varied with sex of the gestating embryo. During gestational week 2, mothers carrying female embryos (N = 10) had higher mean cortisol levels than those carrying male embryos (N = 9) ($t(17)$ = 2.28, P = 0.04).

LIMITATIONS, REASONS FOR CAUTION: Our results are based on a relatively small sample (n = 22) of women. However, our repeated-measures design with an average of 27 \pm 8 (mean \pm SD) data points per woman strengthens the precision of estimates resulting in

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high statistical power. Additionally, our study population's high degree of ethnic and cultural homogeneity reduces the effects of confounders compared with those found in industrialized populations. This higher level of homogeneity also increases our statistical power. However, since there may be small differences in absolute cortisol values among ethnic groups, the social and biological background of our sample may affect the generalizability of our results. General patterns of HPAA activity, however, are expected to be universal across women. Finally, as there is, to the best of our knowledge, no evidence to the contrary, we assumed that urinary cortisol levels reflect HPAA activity and that changes in gonadal steroids across the menstrual cycle do not affect the levels of free cortisol measured in urine.

WIDER IMPLICATIONS OF THE FINDINGS: To our knowledge, this is the first longitudinal profile of basal maternal HPAA activity across the peri-conceptual period. A basic understanding of the normative (basal as opposed to stress-induced) changes in HPAA activity across this period is needed to accurately assess women's stress at this juncture. Importantly, changes in HPAA activity are likely to play a critical role in ovulation, fertilization, implantation, placentation and embryonic programming. Thus, this novel information should aid in the development of interventions aimed at preventing or moderating undesired effects of maternal physiological stress during the peri-conceptual period on reproductive outcomes as well as embryonic development.

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Introduction

Physiological stress has been implicated in a wide range of negative reproductive outcomes in women including decreased fecundability and increased risk of early miscarriage, as well as alterations in the developmental trajectories of their offspring, *in utero* and post-natally (Nepomnaschy *et al.*, 2004, 2006; Jasienska *et al.*, 2006; McGowan *et al.*, 2009; Parker and Douglas, 2010a,b; D'Anna-Hernandez *et al.*, 2011). Thus, stress as a covariate has become ubiquitous in women's reproduction and maternal-child health studies (Hruschka *et al.*, 2005; Sarkar *et al.*, 2013). Yet, our understanding of women's stress physiology is still very limited. Indeed, assessment of women's stress levels is often carried out in the absence of knowledge regarding the longitudinal changes that the stress axis often undergoes, which can lead to the misinterpretation of results.

Basal functioning and responsivity of the stress axis, or hypothalamic-pituitary-adrenal axis (HPAA), changes as women transition between reproductive stages (between phases of the menstrual cycle, from ovarian cyclicity to conception, across gestation, peri-menopause and menopause) (Nepomnaschy *et al.*, 2004, 2006, 2007, 2011, 2012, 2013; Nepomnaschy, 2005). Thus, in order to properly assess women's physiological stress, it is important to first understand how normative ('average' or 'basal' as opposed to stress-induced) HPAA activity varies across reproductive transitions. For several reproductive transitions, however, changes in normative HPAA activity have not been fully described. This is the case for the transition from regular ovarian cyclicity to conception and early gestation.

The earliest available information regarding HPAA activity around the time of conception corresponds to the later weeks of the first trimester (Brien and Darlymple, 1976; Demey-Ponsart *et al.*, 1982; Abou-Samra *et al.*, 1984; Allolio *et al.*, 1990; D'Anna-Hernandez *et al.*, 2011; Jung *et al.*, 2011). The paucity of information on HPAA activity during the weeks immediately preceding and following conception is a consequence of

the designs used in most prenatal studies. For practical reasons, women tend to be recruited after clinical confirmation of their pregnancy; that is at least 6 weeks post-conception. Such recruitment protocols do not allow for the prospective collection of biological specimens before the sixth gestational week.

Producing an accurate description of longitudinal changes in the HPAA's normative (basal) activity in the weeks immediately preceding and following conception is not only important for the correct assessment of women's stress levels, but also for understanding the role of the stress axis in conception, pregnancy maintenance and early embryonic development. Deviations from basal HPAA activity have been linked to lower fecundability (Nepomnaschy *et al.*, 2004; Beehner and Lu, 2013; Whirledge and Cidlowski, 2013), higher risk of miscarriage (Nepomnaschy *et al.*, 2006; Arck *et al.*, 2008; Brunton, 2013) and changes in fetal developmental trajectories (MacLaughlin and McMillen, 2007; Nepomnaschy and Flinn, 2009; Parker and Douglas, 2010a,b). In order to detect changes in HPAA activity (i.e. detect physiological stress), it is first necessary to establish basal function.

To address this important gap in our knowledge, here we present the first longitudinal description of within- and between-women variation in basal urinary cortisol levels during the 12-week period centered on the day of conception (hereafter 'peri-conceptual period'). After describing longitudinal cortisol trajectories, we align them with concurrent reproductive events and embryonic ontogeny to develop hypotheses regarding the potential biological functions of the observed changes.

Cortisol, the most frequently used biomarker of HPAA activity, can be reliably measured in a variety of matrices such as serum, saliva and urine (Pollard, 1997; Altemus *et al.*, 2001; Kanaley and Hartman, 2002; Miller and O'Callaghan, 2002; Padgett and Glaser, 2003; Hruschka *et al.*, 2005). First morning urine (FMU) is a convenient matrix for longitudinal studies of basal HPAA activity as its collection is less invasive than that of blood (Sarkar *et al.*, 2013). Additionally, FMU cortisol levels are less affected by circadian rhythms and diurnal confounders, such as food consumption

and physical activities, than blood and salivary cortisol (Follenius et al., 1987; Pollard, 1995; Lovallo, 2006; Kudielka et al., 2009; Papadimitriou and Priftis, 2009; Mello, 2010). Free cortisol levels measured in FMU reflect levels of free, unbound (bio-active) cortisol circulating in blood (Daughaday and Bremer, 1955; Daughaday et al., 1956; Schedl et al., 1959; Beisel et al., 1964a,b; Lindholm, 1973).

The results presented here should aid in the interpretation of women's cortisol data collected during the peri-conceptual period and, in so doing, contribute to our understanding of the role of the HPA axis in regulating fecundability, pregnancy fate and embryonic development.

Materials and Methods

Ethical approval

The work performed for the present study was approved by the Research Ethics Board at Simon Fraser University (file number: 2010s0557).

Study population and participants

This article is based on data and urinary specimens collected in the context of the 'Society, Environment and Reproduction' (SER) study. SER is a naturalistic, longitudinal study on the interactions between the stress and reproductive axes in women. This study began in the year 2000, and it is based on two neighboring rural Kaqchikel Mayan communities located in the southwest highlands of Guatemala. All women in these two communities who matched the following inclusion profile were invited to participate in SER: parous, living with a co-resident male partner, not pregnant and not using any form of chemical contraceptive method (Nepomnaschy et al., 2004, 2006). A total of 107 women were enrolled and provided informed consent, of which 22 conceived and maintained their pregnancies during the study period through, at least, the first trimester of gestation. The 22 participants were not taking any medication at the time of sample collection. They ranged in age from 19 to 36 years (mean \pm SEM = 27.5 \pm 1.07 years), in body mass index (BMI) from 19.13 to 31.29 kg/m² (mean \pm SEM = 21.88 \pm 0.61) and in parity from 1 to 6 children (mean \pm SEM = 2.9 \pm 0.3 children). All the analyses in this paper are based on the data from these 22 women. The collection of the urinary specimens analyzed for this article took place between November 2000 and October 2001.

Data and bio-specimen collection

We collected a FMU sample three times per week (i.e. every other day with the exception of Sundays) from each participant for the duration of the study or until a clinical pregnancy was detected (i.e. greater than Week 6 of gestation) (Nepomnaschy et al., 2004, 2006). On average participants provided 27 \pm 8 (mean \pm SD) FMU samples (range: 8–35) during the 12-week peri-conceptual period (average rate of compliance with the collection protocol was 75%, i.e. 27 of 36 possible samples). This sampling design allowed us to monitor day-to-day variation in urinary free cortisol levels within- and between-individuals (Nepomnaschy et al., 2011). Upon waking, participants collected their first urinary voids in clean, dry, inert plastic containers provided by our research team the previous night. Local female research assistants visited participants at their homes to obtain each FMU sample on the day it was collected and transported the samples in coolers filled with blue-ice packs to our field station within 4 h of the urinary void. FMU samples were aliquoted into 2 ml cryo-vials and stored at -10°C at the station. Samples were transported on dry ice from the field to our laboratory, where we stored them at -80°C until analyses.

Hormone analyses

We evaluated urinary concentrations of biomarkers of stress, ovulation, conception and pregnancy maintenance. FMU levels of cortisol were measured to assess HPA axis function. FMU estrone-3-glucuronide (E1G) and pregnanediol glucuronide (PdG), urinary estradiol and progesterone conjugates, were quantified to identify the day of ovulation, while human chorionic gonadotropin, beta sub-unit (hCG- β) was used to identify and confirm conception and pregnancy maintenance. All hormone assays were performed in 2012 at the Maternal and Child Health Laboratory at Simon Fraser University, BC, Canada.

We quantified FMU cortisol, hCG and E1G levels using a multiplex enzyme immunoassay (EIA) array (Quansys Biosciences, Logan, UT, USA) that was previously validated by our laboratory (Salvante et al., 2012). FMU PdG levels were quantified using a competitive solid-phase EIA (O'Connor et al., 2003) based on the Quidel anti-PdG monoclonal antibody, clone 330, kindly provided by Dr. Bill Lasley, University of California at Davis, Davis, CA, USA. We ran all assays in duplicate and re-assayed samples for which the coefficient of variation between duplicates was $> 13\%$. All intra- and inter-assay coefficients of variation were within acceptable ranges (Table 1). To account for variability in hydration state, we corrected concentrations for specific gravity using refractometry (Miller et al., 2004; White et al., 2010).

Treatment of outliers (i.e. data points > 2 SD within individuals): (a) if all hormones quantified in a given sample were outside an individual's ranges, we inferred an error in the assay and re-ran the hormone array for that sample. If the re-run yielded similar results, we assumed the sample was unreliable and excluded it from further analyses; (b) if a single hormone value was outside an individual's range, but the rest of the hormone levels were within their expected ranges we assumed that there were no problems with sample or assay quality and, thus, we retained the data point.

FMU cortisol as a biomarker of HPA axis activity

FMU cortisol levels are regularly interpreted as the reflection of adrenal cortisol secretion during the period between the last urinary void before an individual retires for the day and their first morning urinary void (Jerjes et al., 2006; Sarkar et al., 2013). This interpretation has resulted in the use of FMU cortisol, also known as nocturnal urinary cortisol, as a marker of HPA axis activity (Pollard, 1995; Nepomnaschy et al., 2004, 2006, 2011, 2012; Jerjes et al., 2006; Valeggia and Ellison, 2009; o Hartaigh et al., 2012; Sarkar et al., 2013; James et al., 2014). Circulating cortisol is metabolized by cytochrome p450 enzymes into 6beta-hydroxycortisol. Therefore, urinary levels of cortisol reflect the non-metabolized portion of circulating free cortisol. Although the ratio of urinary cortisol:6beta-hydroxycortisol is often used as an indirect measure of CYP3A4 activity, for technical reasons we were unable to assay 6beta-hydroxycortisol in our urine samples. Cytochrome p450 enzymes also metabolize gonadal steroids such as estradiol and progesterone. Thus, if the affinity of this group of enzymes were higher for

Table 1 Hormone assay quality control data.

Urinary metabolite	Intra-assay CV (%)	Inter-assay CV (%)
Cortisol	6.7	12.76
E1G	8.2	9.0
hCG	11.1	13.4
PdG	9.1	9.3

Coefficient of variation (CV), estrone-3-glucuronide (E1G), human chorionic gonadotropin (hCG), pregnanediol glucuronide (PdG).

progesterone and estradiol than for cortisol, it could be argued that regular changes in circulating levels of those gonadal steroids could influence the levels of cortisol measured in urine. There is no evidence, however, for the existence of differences in affinity of cytochrome p450 enzymes for any of those three steroids (Kerlan *et al.*, 1992; Domanski *et al.*, 2000; Bu, 2006). Furthermore, there is no evidence suggesting that changes in circulating levels of gonadal steroids in regular ovarian cycles are associated with variation in urinary free cortisol levels. Specifically, in a recent study we found that the day of the menstrual cycle did not predict urinary cortisol levels during an average 28 day cycle (i.e. between days -14 (follicular phase) through $+14$ (luteal phase), wherein day 0 is the day of ovulation), despite the large predictable fluctuations in both estrogen and progesterone on specific days of the cycle (Nepomnaschy *et al.*, 2011). These results were consistent with other studies based on salivary cortisol (Kudielka and Kirschbaum, 2003; LeRoux *et al.*, 2014), which also reflects levels of circulating free cortisol (Lewis, 2006; Gröschl, 2008). Therefore, for this study we assume that urinary cortisol levels reflect HPA activity and that changes in gonadal steroids across the menstrual cycle do not affect levels of free cortisol measured in urine.

Inclusion criteria and the alignment of ovarian cycles

To avoid the influence of early pregnancy loss, which has been linked to increased cortisol levels (Nepomnaschy *et al.*, 2006), on cortisol trajectories we excluded conceptive cycles that resulted in miscarriages during the first trimester. Given the importance of accurately ascertaining day of ovulation for our analyses, we also excluded pregnancies that were conceived before women enrolled in the study or while they were temporarily absent from it.

Conceptive and non-conceptive cycles were aligned on the day of ovulation for an appropriate comparison of cortisol trajectories during the follicular phase (Nepomnaschy *et al.*, 2004, 2006). Alignment of conceptive and non-conceptive cycles on the day of ovulation is more accurate than alignment on the first day of the cycle due to potential differences among women in the length of the follicular phase. Furthermore, the lack of a mid-luteal progesterone peak in conceptive cycles, which do not contain complete luteal phases, prevents their alignment using this hormonal milestone.

Statistical analyses

All statistical analyses were performed using R (Version 3.0.1, 2012, R Development Core Team, Vienna, Austria). To normalize the data distribution, we log transformed (base 10) all specific gravity-corrected FMU cortisol values (referred to simply as cortisol hereafter) (Keene, 1995). While the number of participants included in our analyses is modest ($n = 22$), our repeated-measures design includes an average of 27 ± 8 (mean \pm SD) data points per participant. When analyzed using mixed-effects modeling, as we have used in this study, the heteroscedasticity and dependence among the repeated measurements is incorporated through random effects, which increases the precision of estimates and results in higher statistical power (Burton *et al.*, 1998). A P value < 0.05 was considered as being significant.

Average cortisol trajectory across the peri-conceptual period

A graphic representation of the average cortisol trajectory was developed using the locally-weighted scatterplot smoothing (LOESS) 'xyplot' function in the 'lattice' package in R. LOESS is a non-parametric regression smoothing method that fits local polynomial regressions to localized subsets of the data and joins them together to build a smooth function describing the deterministic variation in the data (Cleveland, 1979). The objective is to identify the estimated curve that best represents the population curve. LOESS curves allow for the visualization of the underlying structure of the longitudinal hormone profiles; however, no confidence intervals and tests on individual coefficients associated with the time trend can be obtained. To that aim

we used linear mixed-effect models with regression splines, which can account for both intra- and inter-individual variation in cortisol through random effects (Burton *et al.*, 1998; Edwards *et al.*, 2006). Mixed-effects models were, thus, used to isolate changes in cortisol as predicted by day and reproductive status (e.g. pre- versus post-conception, days from conception etc.) (Burton *et al.*, 1998; Hruschka *et al.*, 2005). We included maternal age, BMI and parity as covariates in the spline analysis as these factors have previously been shown to influence maternal cortisol secretion during pregnancy (D'Anna-Hernandez *et al.*, 2012; Peer *et al.*, 2013; Conde and Figueiredo, 2014; de Vries *et al.*, 2014).

Preconception versus post-conception cortisol levels

To compare cortisol levels between the pre- and post-conception periods, we fit the following linear mixed effects model

$$\log_{10}(\text{cortisol}_{ij}) = \beta_0 + \beta_1 x_{ij} + b_{0i} + b_{1i} x_{ij} + e_{ij}$$

where x_{ij} indicates whether the sample was collected pre- ($x_{ij} = 0$) or post-conception ($x_{ij} = 1$), β_0 represents the average preconception log cortisol, β_1 is the average change in log cortisol post-conception, b_{0i} and b_{1i} are individual specific random effects for preconception values and change in post-conception values, respectively and e_{ij} is random error due to intra-individual variation.

To evaluate the amount of inter-individual variation in the change in cortisol from pre- to post-conception, we conducted a likelihood ratio test (LRT) to compare the fit of two different linear mixed effects models: one including the random effect term $b_{1i} x_{ij}$ and another without it. The model that included the $b_{1i} x_{ij}$ term allowed individuals to vary from one another in the extent to which their post-conception cortisol changed from their preconception values.

Directional trends in cortisol across the peri-conceptual period

The LOESS curve (Fig. 1) showed that cortisol's trajectory varied dynamically. Thus, we decided to evaluate the magnitude and significance of these directional trends across the whole peri-conceptual period. To that aim, we used a mixed-effects model with regression splines ('lme' function in the 'nlme' package in R). We began with a mixed model containing maternal BMI, age and parity as fixed effects and cubic regression splines with weekly knots (i.e. potential directional changes at Days -35 , -28 , -21 , -14 , -7 , 0 , 7 , 14 , 21 , 28 and 35) as both fixed and random effects. The Akaike information criterion, Bayesian information criterion and log-likelihood values from the fitted reduced models were used to choose the optimal spline basis and knots. The best-fitted model had fixed linear regression splines with knots at Days $t^* = -7$, 0 , 28 and 35 and a random linear time trend:

$$\begin{aligned} \log_{10}(\text{cortisol}_{ij}) = & \beta_0 + \beta_1 t + \beta_2 * \text{BMI}_i + \beta_3 * \text{PAR}_i + \beta_4 * \text{AGE}_i \\ & + \gamma_1 (t - (-7))_+ + \gamma_2 (t - 0)_+ + \gamma_3 (t - 28)_+ \\ & + \gamma_4 (t - 35)_+ + \alpha_{i0} + \alpha_{i1} t + e_{ij}, \end{aligned}$$

where $(t - t^*)_+$, known as a truncated line function, equals $(t - t^*)$ when $t > t^*$ and equals zero when $t \leq t^*$. The slope of the first period, β_1 , was statistically compared with zero (0), while the slope of each successive period was compared with the slope of the preceding period through coefficients γ 's. The random linear trend, α_{i1} , allows between-individual variation in cortisol slopes within each period.

This model allows for a more detailed description of the cortisol pattern across the peri-conceptual period because the coefficients associated with the linear time trend variable for each period can be estimated and tested. For the ease of presentation, the final model is equivalently written as separate linear equations for each of the five periods defined by the knots.

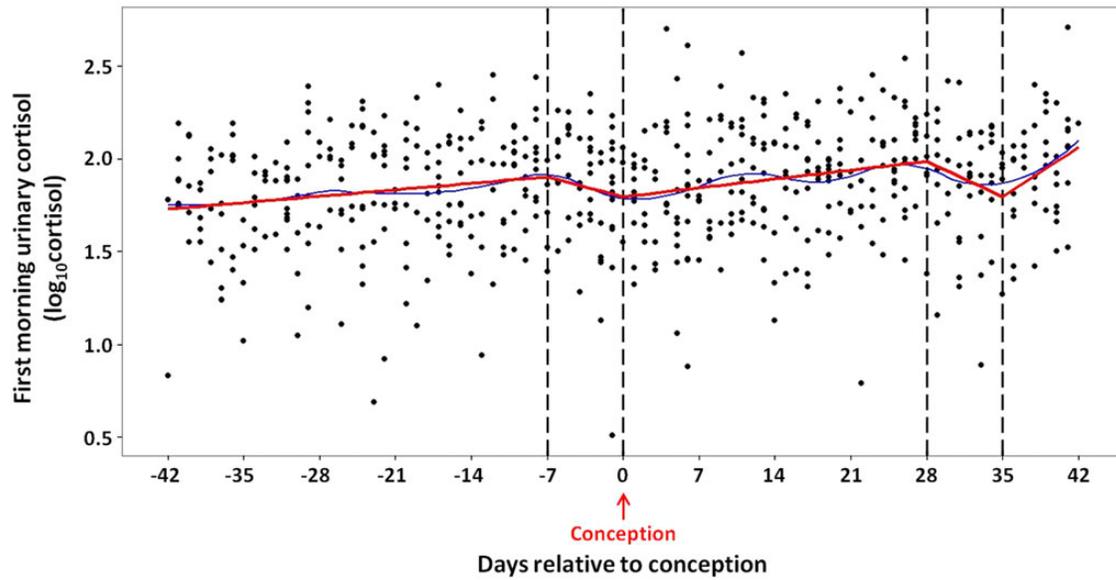


Figure 1 Locally weighted scatterplot smoothing curve (blue line) and fitted average (red line) for \log_{10} -transformed, first-morning urinary cortisol levels in women across the peri-conceptual period (± 6 weeks around conception) of 22 pregnancies. Cortisol was corrected for specific gravity, then \log_{10} -transformed. Day 0 is the day of conception. The vertical dashed lines at Days -7 , 0 , $+28$ and $+35$ represent ‘knots’ (i.e. inflection points) at which the trajectory of cortisol changes.

1) Day -42 through Day -7 :

$$\log_{10}(\text{cortisol}_{ij}) = \beta_0 + \beta_1 t + \beta_2 * \text{BMI}_i + \beta_3 * \text{PAR}_i + \beta_4 * \text{AGE}_i + \alpha_{i0} + \alpha_{i1} t + e_{ij},$$

2) Day -6 through Day 0 :

$$\log_{10}(\text{cortisol}_{ij}) = (\beta_0 - \gamma_1(-7)) + (\beta_1 + \gamma_1)t + \beta_2 * \text{BMI}_i + \beta_3 * \text{PAR}_i + \beta_4 * \text{AGE}_i + \alpha_{i0} + \alpha_{i1} t + e_{ij},$$

3) Day $+1$ through Day $+28$:

$$\log_{10}(\text{cortisol}_{ij}) = (\beta_0 - \gamma_1(-7) - \gamma_2(0)) + (\beta_1 + \gamma_1 + \gamma_2)t + \beta_2 * \text{BMI}_i + \beta_3 * \text{PAR}_i + \beta_4 * \text{AGE}_i + \alpha_{i0} + \alpha_{i1} t + e_{ij},$$

4) Day $+29$ through Day $+35$:

$$\log_{10}(\text{cortisol}_{ij}) = (\beta_0 - \gamma_1(-7) - \gamma_2(0) - \gamma_3(28)) + (\beta_1 + \gamma_1 + \gamma_2 + \gamma_3)t + \beta_2 * \text{BMI}_i + \beta_3 * \text{PAR}_i + \beta_4 * \text{AGE}_i + \alpha_{i0} + \alpha_{i1} t + e_{ij},$$

5) Day $+36$ through Day $+42$:

$$\log_{10}(\text{cortisol}_{ij}) = (\beta_0 - \gamma_1(-7) - \gamma_2(0) - \gamma_3(28) - \gamma_4(35)) + (\beta_1 + \gamma_1 + \gamma_2 + \gamma_3 + \gamma_4)t + \beta_2 * \text{BMI}_i + \beta_3 * \text{PAR}_i + \beta_4 * \text{AGE}_i + \alpha_{i0} + \alpha_{i1} t + e_{ij},$$

where β_0 is cortisol at the beginning of the study (Day -42), β_2 is associated with maternal BMI, β_3 is associated with maternal parity, β_4 is associated with maternal age, β_1 is the slope of FMU cortisol during the first period, $\beta_1 + \gamma_1$ is the slope of cortisol during the second period, $\beta_1 + \gamma_1 + \gamma_2$ is the slope of cortisol during the third period, $\beta_1 + \gamma_1 + \gamma_2 + \gamma_3$ is the slope of cortisol during the fourth period, $\beta_1 + \gamma_1 + \gamma_2 + \gamma_3 + \gamma_4$ is the slope of cortisol during the fifth period, α_{i0} is the individual-specific random intercept, α_{i1} is the individual-specific random slope to account for between-individual variation, and e_{ij} is the random error term. The slope of the first period is compared with slope = 0, while the slope of each subsequent period is compared with the slope of the preceding period.

Exploratory analyses

We conducted two sets of exploratory analyses on relevant topics for which we had a smaller sample size: (i) a comparison of pre-ovulatory cortisol trajectories between conceptive and non-conceptive cycles and (ii) a comparison of cortisol trajectories during the early post-conception period between women carrying female and male embryos. Given the size of our sample for these analyses (see below), our goal is not to draw conclusions but to promote research and scientific discussions on these two critical topics.

Cortisol trajectories leading up to ovulation in conceptive versus non-conceptive cycles. Of the 22 women who conceived pregnancies, 12 also provided data from complete non-conceptive ovarian cycles. We used this small sample to explore potential differences in pre-ovulatory cortisol trajectories between conceptive and non-conceptive cycles. Using a mixed-effects model we compared their cortisol levels starting at Day -20 and ending at ovulation (Day 0):

$$\log_{10}(\text{cortisol}_{ijk}) = \beta_0 + \beta_1 t_{ijk} + \beta_2 X_{ij} + \beta_3 t_{ijk} X_{ij} + \beta_4 t_{ijk}^2 + \beta_5 t_{ijk}^2 X_{ij} + b_i + \alpha_{ij} + e_{ij}$$

Table II Weekly geometric mean first-morning urinary cortisol during the peri-conceptual period (weeks relative to conception; Day 0 = conception).

Study week	Day range	Number of women that provided samples	Geometric mean (ng/ml)	SD
-6	Days -42--36	17	58.14	36.00
-5	Days -35--29	17	51.27	33.91
-4	Days -28--22	18	65.39	42.78
-3	Days -21--15	18	70.91	50.07
-2	Days -14--8	20	71.52	44.80
-1	Days -7--1	22	69.10	41.26
1	Days 0-6	22	64.43	46.76
2	Days 7-13	22	82.57	43.63
3	Days 14-20	20	74.55	47.20
4	Days 21-27	20	86.96	47.29
5	Days 28-34	18	76.34	45.04
6	Days 35-42	18	89.29	46.76

where t_{ijk} is the k th day in the cycle with respect to the day of ovulation ($t_{ijk} = 0$) for woman i in cycle j , $X_{ij} = 1$ indicates that woman i is in a conceptive cycle, where the j th cycle is the conceptive cycle, β_0 is the average log cortisol on the day of ovulation in a non-conceptive cycle, $\beta_1 + 2\beta_4 t_{ijk}$ represents the average rate of change in log cortisol in the non-conceptive cycles, β_2 is the average difference between log cortisol levels at ovulation when women are in a conceptive cycle versus when women are in a non-conceptive cycle, β_3 and β_5 represent the difference between the quadratic trends of conceptive and non-conceptive cycles, $(\beta_1 + \beta_3) + 2(\beta_4 + \beta_5)t_{ijk}$ represents the average rate of change in log cortisol levels in the conceptive cycles, b_i is the random woman effect, α_{ij} is the random cycle effect for a woman and e_{ij} is the random measurement error effect.

Cortisol trajectories during the early post-conceptual period by sex of the embryo. Sex data were available for 19 of the 22 pregnancies in this study. We used t -tests to evaluate the relationship between the sex of the developing embryo and maternal cortisol during each of the first 6 weeks of gestation.

Results

Cortisol fluctuated significantly across the 12-week study period of the 22 pregnancies studied (Table II; Fig. 1). Overall, cortisol increased an average of 2.6 ng/ml per week across the entire peri-conceptual period, from 58.14 ± 36.00 ng/ml (geometric mean \pm SD) 6 weeks prior to conception to 89.29 ± 46.76 ng/ml 6 weeks after conception (Table II). Maternal age, parity and maternal BMI were included in the analysis of cortisol trajectory during the peri-conceptual period as potential confounders. The multivariate model showed that, on average, cortisol levels increased 7.6% per year of maternal age ($P = 0.0028$). Parity, however, was negatively correlated with cortisol levels such that each additional offspring was associated with a 18.4% decrease in cortisol levels on average ($P = 0.0077$). We found no association between maternal BMI and cortisol levels ($P = 0.9375$).

In the set of conceptive cycles analyzed ($n = 22$), cortisol was an average of 18.85% higher in the 6 weeks following conception than in the 6 weeks preceding conception ($\beta_1 = 0.075 \pm 0.033$ (coefficient \pm SE), $P = 0.022$). The magnitude of the increase between the pre- and post-

conception periods, however, varied significantly between women (LRT statistic = 8.0017, $P = 0.005$ based on a mixed χ^2 distribution). The linear spline mixed effects models revealed significant differences between the population slopes (Table III; Fig. 1). From Days -42 to -7, cortisol followed a mild linear increasing trajectory (1.36% per day; $P = 0.0007$). Then, after peaking at Day -7, cortisol decreased ($P = 0.0013$ compared with the trajectory observed during the preceding period) at a rate of 4.02% per day until the day of conception (Day 0). After conception, cortisol started to slowly increase at a rate of 1.73% per day for 4 weeks ($P = 0.0008$ compared with the preceding period). Between Days 29 and 35 after conception, the previously increasing trajectory turned into a decline ($P = 0.0002$) at a rate of 6.60% per day. After that 1 week decline period, cortisol's trajectory changed once again to a strong increase ($P = 0.0020$) at a rate of 8.86% per day between days 36 and 42 post-conception, the last week of the study period (Table III; Fig. 1).

Twelve women provided samples from both conceptive and non-conceptive cycles. The analysis of those samples revealed that pre-ovulation cortisol trajectories of conceptive and non-conceptive cycles from the same women were marginally different ($P = 0.0633$). While in their conceptive cycles cortisol followed a parabolic trajectory, increasing from Days -20 to -6, then decreasing until the day of ovulation (rate of cortisol change = $-0.02 - 0.0035t$ ng/ml/day; Table IV), in their non-conceptive cycles cortisol's trajectory was rather flat and time independent. Another interesting source of variation in maternal cortisol levels was the sex of the gestating embryo. During gestational week 2, mothers carrying female embryos ($N = 10$) had higher mean cortisol levels than those carrying male embryos ($N = 9$) ($t(17) = 2.28$, $P = 0.04$).

Discussion

To our knowledge this article presents the first description and analysis of women's basal peri-conceptual cortisol trajectories. Our longitudinal evaluation of this period uncovered that: (a) FMU cortisol excretion varies significantly across the peri-conceptual period; (b) in conceptive cycles ovulation is preceded and immediately followed by an overall

Table III Linear spline mixed effect model for \log_{10} -transformed first-morning urinary cortisol trajectory in women across the peri-conceptual period, estimated regression coefficients (fixed effects).

Fixed effects	Coefficient	SE	P-values
β_0 : cortisol at Day -42 (intercept)	1.3000	0.3245	0.0001
β_1 : cortisol slope during the first period (from Day -42 to Day -7)	0.0059	0.0017	0.0007
β_2 : maternal BMI	0.0009	0.0112	0.9375
β_3 : parity	-0.0881	0.0292	0.0077
β_4 : maternal age	0.0317	0.0091	0.0028
γ_1 : difference in cortisol slope between the first period and the second period (from Day -6 to Day 0) ^a	-0.0237	0.0074	0.0013
γ_2 : difference in cortisol slope between the second period and the third period (from Day +1 to Day +28) ^b	0.0253	0.0075	0.0008
γ_3 : difference in cortisol slope between the third period and the fourth period (from Day +29 to Day +35) ^c	-0.0371	0.0100	0.0002
γ_4 : difference in cortisol slope between the fourth period and the fifth period (from Day +36 to Day +42) ^d	0.0665	0.0214	0.0020

^aCortisol slope during the second period (from Day -6 to Day 0) = $(\beta_1 + \gamma_1)$.

^bCortisol slope during the third period (from Day +1 to Day +28) = $(\beta_1 + \gamma_1 + \gamma_2)$.

^cCortisol slope during the 4th period (from Day +29 to Day +35) = $(\beta_1 + \gamma_1 + \gamma_2 + \gamma_3)$.

^dCortisol slope during the fifth period (from Day +36 to Day 42) = $(\beta_1 + \gamma_1 + \gamma_2 + \gamma_3 + \gamma_4)$.

Table IV First-morning urinary cortisol trajectories before ovulation in conceptive versus non-conceptive cycles in women.

Effect	Coefficient	SE	P-values
β_0	1.7902	0.0650	<0.0001
β_1	0.0002	0.0114	0.9867
β_2	0.0721	0.0897	0.4296
β_3	-0.0202	0.0193	0.2972
β_4	0.0002	0.0006	0.7530
β_5	-0.0019	0.0010	0.0633

β_0 : average log cortisol on the day of ovulation in a non-conceptive cycle.

$\beta_1 + 2\beta_4$: rate of change in log cortisol in non-conceptive cycles.

β_2 : difference in cortisol at ovulation between conceptive and non-conceptive cycles.

β_3 and β_5 : difference in quadratic trends in cortisol between conceptive and non-conceptive cycles.

$(\beta_1 + \beta_3) + 2(\beta_4 + \beta_5)$: rate of change in log cortisol in conceptive cycles.

increase in cortisol excretion, which appears not to be the case for non-conceptive cycles and (c) this overall increasing trajectory is interrupted by two transitory drops in cortisol levels: the first one during the week preceding conception and the second one during the fifth gestational week. An exploratory analysis suggests that basal post-conception cortisol trajectories may differ between women carrying male and female embryos.

This novel information highlights the need for further research on HPA activity during the peri-conceptual period. This knowledge is paramount to accurately assess women's stress and, importantly, to develop interventions to prevent or moderate the undesired effects of physiological stress on women's health, reproductive outcomes and embryonic development.

Preconceptional period

Our observation that conceptive cycles were preceded by at least 5 weeks of a sustained increase in basal FMU cortisol is in line with

earlier reports (Ellison and Valeggia, 2003; Valeggia and Ellison, 2009). Their analyses of monthly urine specimens from a group of women transitioning from post-partum amenorrhoea to the resumption of ovarian cyclicity showed an increase in c-peptide and basal cortisol in the months prior to the first ovarian cycle (Ellison and Valeggia, 2003; Valeggia and Ellison, 2009). Rises in c-peptide production indicate an increase in insulin secretion, a critical mediator of energy intake and storage for specific tissues (Polonsky et al., 1988; Emery Thompson and Knott, 2008). Cortisol increases trigger gluconeogenesis, leading to higher levels of circulating glucose. Ellison and Valeggia argue that this increase in circulating levels of metabolic energy is important for the resumption of ovarian function after post-partum amenorrhea. Consistent with their hypothesis, the increase in basal cortisol we observed preceding conceptive cycles but not non-conceptive ones in our population supports the notion that fecundability may be linked to levels of available energy. Our findings are also compatible with an important body of literature linking stress challenges to reproductive suppression. Psychosocial, energetic and health stress challenges trigger the activation of the HPA leading to changes in the allocation of available metabolic energy to tissues involved in the stress response (Selye, 1976; McEwen and Wingfield, 2003; McEwen, 2004; Nepomnaschy et al., 2004, 2006; Landys et al., 2006; Uhart et al., 2006). As metabolic energy is finite, its re-allocation is achieved at the expense of other metabolic tasks that can be momentarily postponed such as reproduction (Ellison and Lager, 1986; Bentley et al., 1999; Jasienska, 2001, 2003; Valeggia and Ellison, 2003, 2009; Jasienska and Ellison, 2004; Nepomnaschy et al., 2004, 2006, 2007; Vitzthum et al., 2009).

Importantly, in our sample, the increasing basal cortisol trajectory was interrupted by a period of significant decline during the week preceding conception. This decline may be critical for fecundability. The week preceding conception corresponds to the follicular phase of the ovarian cycle, a critical period for follicle maturation, ovulation and conception. Indeed, elevated levels of cortisol and salivary alpha-amylase, another stress biomarker, during this period have both been associated with lower chances of conception (Louis et al., 2011; Lynch et al., 2014). Stress-induced HPA activation during this week has been linked to deleterious changes in the secretion of gonadotrophins and gonadal

steroids which can affect follicle maturation (Rivier and Vale, 1990; Chrousos *et al.*, 1998; Nepomnaschy *et al.*, 2007; Zhang *et al.*, 2011). Thus, it is plausible that the observed decrease in basal cortisol levels during the week preceding ovulation of conceptive cycles plays a protective role in the processes involved in follicle maturation, ovulation and conception.

Post-conceptual period

Until the present study, maternal cortisol trajectories post-conception had only been described for later stages of gestation. The lack of

information regarding trajectories earlier during gestation is linked to logistical limitations of study designs as most prenatal studies recruit women once they learn they are pregnant, ~6–8 weeks post-conception (Table V). Later gestational stages (less than gestational week 8) are characterized by a constant increase in basal cortisol levels (Brien and Darlymple, 1976; Demey-Ponsart *et al.*, 1982; Bustamante and Crabbé, 1984; Allolio *et al.*, 1990; Brunton *et al.*, 2008; Entringer *et al.*, 2010; D'Anna-Hernandez *et al.*, 2011; Jung *et al.*, 2011). This hypercortisolemic state has been argued to constitute a response to the energetic demands imposed by fetal growth (Entringer *et al.*, 2010; Jung *et al.*, 2011), performing regular activities while carrying the extra

Table V Human studies that include cortisol data from the first trimester of pregnancy.

Earliest sample collection period (weeks post-conception)	Sample matrix and collection design	Control group	First significant increase in cortisol relative to control group	Contains peri-conceptual period?	Reference
Weeks 3–11	A single plasma sample per woman	Non-pregnant women	Total cortisol: first trimester Free cortisol: second trimester	Single samples collected from weeks 3–11 were analyzed together. Thus, this study does not provide specific information about peri-conceptual cortisol values or trajectories	(Abou-Samra <i>et al.</i> , 1984)
Weeks 9–12	17 saliva samples per woman, per day, every 4 weeks until parturition	Non-pregnant women	Average salivary cortisol from 0700 h to 2300 h: Weeks 25–28	No	(Allolio <i>et al.</i> , 1990)
Weeks 12–15	One plasma sample per woman, every 4 weeks until parturition	Non-pregnant women	Total cortisol: Weeks 12–15 Free cortisol: Weeks 24–27	No	(Brien and Darlymple, 1976)
Week 15 (hair) (integrated measure of cortisol from previous 3 months = Weeks 3–15) Weeks ~12 to ~16 (saliva)	One hair sample per woman at Week 15 and Week 36 and at Week 12 post-partum Three saliva samples per woman, per day, for 3 consecutive days, once in first trimester	Same women during the post-partum period	Hair cortisol: Week 36 sample (reflects cortisol from Weeks 24–36) Salivary cortisol (area under the curve ground): Weeks ~12 to ~16	This study does not provide specific information about peri-conceptual cortisol. Hair cortisol provides an integrated measure of cortisol secretion for the 3 months before sample collection, including late first and early second trimester values. No for the saliva samples	(D'Anna-Hernandez <i>et al.</i> , 2011)
Weeks 3–7	A single serum sample per woman	Non-pregnant women	Total cortisol: Weeks 8–12 Unbound cortisol: Weeks 18–22	Single samples collected from weeks 3–7 were analyzed together. Thus, this study does not provide specific information about peri-conceptual cortisol values or trajectories	(Demey-Ponsart <i>et al.</i> , 1982)
Weeks 8–14	One plasma sample and one 24-h urine sample per woman, per trimester and at 2–3 months post-partum	Non-pregnant women	Total plasma cortisol: first trimester 24-h urine: first trimester Free plasma cortisol: second trimester	No	(Jung <i>et al.</i> , 2011)
Weeks 1–3	Three FMU samples per week (collected every other day) per woman for first 3 weeks of gestation	Unsuccessful (miscarried) pregnancies	Cortisol was higher in unsuccessful (miscarried) pregnancies than in successful pregnancies	Yes	(Nepomnaschy <i>et al.</i> , 2006)

weight and volume associated with pregnancy (Butte and King, 2005; Forsum and Löf, 2007) and the development of energetic reserves in preparation for nursing (Butte and King, 2005).

Our results suggest that the increase in basal cortisol levels actually begins earlier, at the moment of conception. The average rate of basal cortisol increase observed right after conception is significantly higher than the one characterizing the 6 weeks preceding it. Future studies should explore the function(s) of this early post-conceptual increase in basal cortisol. Increases in basal cortisol levels during this period may provide the extra metabolic energy required for the processes of implantation and placentation (Clapp et al., 1988; Butte and King, 2005; Forsum and Löf, 2007).

The post-conceptual increase in cortisol may also contribute to the same processes that lead to hypercortisolemia later during gestation; the allocation and storage of energy to specific tissues involved in sustaining pregnancy later on, the delivery and nursing of the baby and post-natal maternal care. Nonetheless, the basal cortisol levels we observed during the first 6-week post-conception were clearly lower than those reported for later gestational stages, and the rate of increase was more gradual (Brien and Darlrymple, 1976; Demey-Ponsart et al., 1982; Bustamante and Crabbé, 1984; Allolio et al., 1990; Brunton, et al., 2008; Entringer et al., 2010; D'Anna-Hernandez et al., 2011; Jung et al., 2011). This slow post-conceptual increase may be explained by a trade-off between the emerging energy demands of early gestation (Clapp et al., 1988; Butte and King, 2005) and the risks associated with elevated glucocorticoid exposure. Elevated basal cortisol after conception may increase the risk of early miscarriage and could affect the processes of implantation, placentation and the earliest stages of embryonic development (Barnes and Adcock, 1993; Estelles et al., 1994; Barnes, 1998; Jauniaux et al., 2003; Michael and Papageorgiou, 2008). Implantation involves inflammatory processes that mediate structural changes in the endometrium preceding implantation (Granot et al., 2012; Van Sinderen et al., 2012). Thus, as cortisol is anti-inflammatory, maintaining relatively low basal cortisol levels during this period may be paramount to the successful remodeling of the endometrium and implantation (Barnes and Adcock, 1993; Barnes, 1998). Cortisol also plays an important role in placentation. It acts as a modulator of regulatory pathways involved in the invasion of the placental trophoblast (Jones et al., 1989; Ma et al., 2002; Nicholson et al., 2004; Kalantaridou et al., 2007; Michael and Papageorgiou, 2008). Excess cortisol exposure during the formation of the placenta may result in shallow trophoblast invasion. In turn, shallow trophoblast invasion has been linked to a variety of complications such as premature onset of maternal-placental circulation, leading to elevated oxidative stress and increased risk of miscarriage, intrauterine growth restriction and pre-eclampsia (Estelles et al., 1994; Jauniaux et al., 2003; Michael and Papageorgiou, 2008).

The arguments above may also help explain the significant decline in basal cortisol we observed during Week 5 post-conception. Later during gestation the fetus is partially protected from exposure to high maternal cortisol levels (both basal and stress-induced) by the placental expression of 11 beta-hydroxysteroid dehydrogenase 2 (11 β -HSD2), an enzyme that converts cortisol into inactive cortisone (Benediktsson et al., 1997). If the amount of placental 11 β -HSD2 produced is not sufficient to counter an increase in maternal cortisol, the excess glucocorticoid reaching the fetus can have serious effects on its development. Depending on the timing of the exposure and its magnitude, high maternal cortisol could lead to a broad spectrum of negative outcomes ranging

from the alteration of fetal growth rates to an increase in the risk of cardiovascular disease in the adult (Barker, 1990; Gitau et al., 1998; Barker and Bagby, 2005; Reynolds, 2010, 2013; Duthie and Reynolds, 2013). Importantly, 11 β -HSD2 may not be present to protect the embryo during the weeks immediately following conception. Indeed, the earliest that 11 β -HSD2 has been detected is 6 weeks post-conception (Shams et al., 1998; McTernan et al., 2001). Thus, as basal cortisol increases after conception, the days immediately preceding the onset of 11 β -HSD2 production may be particularly sensitive to high glucocorticoid levels. Therefore, it is possible that the decline in basal maternal cortisol levels during gestational week 5 may be part of a mechanism protecting the embryo from high glucocorticoid levels at this critical developmental juncture.

Covariates associated with cortisol levels

In our sample, FMU basal cortisol levels were associated with maternal age, parity and sex of the embryo. Counterintuitively, age and parity presented opposite effects. Basal cortisol levels were positively associated with age but negatively associated with parity. Previous studies based on plasma and salivary cortisol have reported modest increases in its basal levels with age (salivary cortisol: (Heaney et al., 2012); plasma cortisol: (Seeman et al., 1995; Kern et al., 1996; Lupien et al., 1996)), but others have reported declines (plasma cortisol: (Lupien et al., 1996)) or no change (plasma cortisol: (Leblhuber et al., 1993; Born et al., 1995; Thomas et al., 1999; de Bruijn et al., 2002)) with increasing age. At this point, it is unclear why these studies yield opposite results even when using the same matrices (for a discussion of this issue, see Lupien et al., 1996). Our results suggest that these inconsistencies may be explained, at least in part, by a potential buffering effect of parity on basal cortisol. Consistent with our findings, a previous study reported that first-time mothers presented more prominent increases in basal cortisol between the second and third trimesters of pregnancy than second-time mothers (Conde and Figueiredo, 2014). Nonetheless, further studies will be needed to confirm these findings and test the existence of the proposed buffering effect of parity on the increase in basal cortisol levels with age.

Sex of the embryo was another factor associated with maternal cortisol levels. To our knowledge, this is the first study to evaluate this relationship. Women carrying female embryos presented higher basal cortisol levels during post-conceptual week 2 than those carrying males. This observation is based on a small sub-sample of pregnancies and, thus, needs to be confirmed by larger studies. Furthermore, this result was not in line with our *a priori* predictions. Males are, on average, more energetically expensive to produce than females (Clutton-Brock et al., 1984; Byrne and Warburton, 1987; Kline and Stein, 1987; Damjanovic et al., 2009; Catalano et al., 2013). Therefore, women carrying males would be expected to present higher metabolic rates and, thus, higher basal cortisol levels than those carrying females (Damjanovic et al., 2009). Our results, however, may be explained by a sex bias in early miscarriages. The risk of miscarriage is highest immediately after conception (Kline and Stein, 1987; Catalano et al., 2013), and males are more likely to be miscarried than females (Byrne and Warburton, 1987; Wells, 2000). This is particularly so in response to stress (Chason et al., 2012; Davis et al., 1998, 2007; Song, 2012; Catalano et al., 2013), which is associated with increases in cortisol. Thus, if mothers who had high overall (i.e. both basal and stress-induced) cortisol levels were more likely to lose males

than females, then our sample of male-carrying mothers would be biased toward those who had lower overall cortisol levels.

Summary and future directions

In summary, our results suggest that basal HPA activity varies dynamically across the peri-conceptual period. In our sample, conceptive cycles were characterized by an overall increase in FMU cortisol excretion spanning the full 12 weeks of the study period. This overall increasing trajectory was interrupted twice by 1-week declines: once during the week preceding ovulation and the other during the fifth week of gestation. FMU cortisol levels were associated with women's age, parity and, post-conception, the sex of their embryo.

We propose that the overall increase in basal cortisol secretion, suggested by its increase in FMU excretion, may mediate a shift in energy allocation from regular metabolic maintenance toward reproductive function. Preconception, the increase in circulating metabolic energy would be allocated to reproductive function, increasing the chance of conception. Post-conception, that energy would be allocated toward meeting the energetic demands of early gestation and storage for use later in pregnancy and post-natal care. Since high overall cortisol levels may elevate the risk of miscarriage and affect embryonic development, the gradual nature of basal cortisol's largely increasing trajectory and the 2 week-long declines observed during the study period may reflect a trade-off between meeting pregnancy's energetic needs and minimizing overall cortisol exposure during specific windows of vulnerability.

Future studies conducted in other populations and including larger sample sizes will be required to confirm our findings, assess their generalizability and evaluate the biological functions of the observed changes in HPA activity across the peri-conceptual period. Fully understanding normative longitudinal variation in HPA activity across reproductive transitions is vital to comprehend how deviations from normative patterns can influence maternal health and wellbeing, fecundability, risk of miscarriage and fetal programming as well as to develop interventions aimed at preventing these deviations or moderating their effects.

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Authors' roles

P.A.N. initiated and designed the study. P.A.N. collected the samples. K.G.S., C.P. and C.K.B. performed the laboratory analyses of the samples. H.M., J.C.B., L.W. and L.Z. analyzed the data. All authors interpreted the data. K.G.S. and P.A.N. wrote the manuscript with input from the other authors.

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Conflict of interest

None declared.

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