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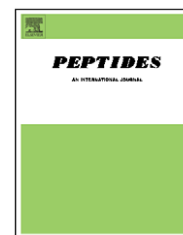
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Elevated maternal cortisol early in pregnancy predicts third trimester levels of placental corticotropin releasing hormone (CRH): Priming the placental clock

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ABSTRACT

The purposes of this study were to determine the intervals when placental corticotrophic-releasing hormone (CRH) was most responsive to maternal cortisol. A sample of 203 women each were evaluated at 15, 19, 25 and 31 weeks gestation and followed to term. Placental CRH and maternal adrenocorticotropin hormone (ACTH), B-endorphin and cortisol were determined from plasma. CRH levels increased faster and were higher in women who delivered preterm compared with women who delivered at term ($F_{3,603} = 5.73$, $p < .001$). Simple effects indicated that CRH levels only at 31 weeks predicted preterm birth ($F_{1,201} = 5.53$, $p = .02$). Levels of cortisol were higher in women who delivered preterm at 15 weeks gestation ($F_{1,201} = 4.45$, $p = .03$) with a similar trend at 19 weeks gestation. Hierarchical regression suggested that the influence on birth outcome of maternal cortisol early in pregnancy was mediated by its influence on placental CRH at 31 weeks. Elevated cortisol at 15 weeks predicted the surge in placental CRH at 31 weeks ($R = .49$, $d.f. = 1,199$, $F_{\text{change}} = 61.78$, $p < .0001$). Every unit of change in cortisol ($\mu\text{g/dl}$) at 15 weeks was associated with a 34 unit change of CRH (pg/ml) at 31 weeks. These findings suggested that early detection of stress signals by the placenta stimulated the subsequent release of CRH and resulted in increased risk for preterm delivery.

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Remarkable surveillance and response systems have evolved and are conserved so that many species from the desert dwelling Western Spadefoot tadpole to the human fetus can detect threats to survival and adjust their developmental trajectory [3,40]. Rapidly evaporating pools of desert water result in elevation of a stress hormone, corticotrophic-releasing hormone (CRH), in the pathway between the brain and the pituitary gland (median eminence) of the tadpole,

precipitating metamorphic climax to escape imminent peril [7,8]. If the CRH response is blocked during environmental desiccation, then the rate of development is arrested and the tadpole's survival is compromised.

The human fetus has evolved similar mechanisms to acquire information about the environment that guide its development. The human placenta is both a sensory and effector organ that incorporates and transduces information

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from its maternal host environment into the fetal developmental program. It has receptors and expresses the genes for major stress systems, including the endocrine system and specifically CRH [10,11,20,23,28,34,35,48]. Placental CRH production increases dramatically over the course of normal human gestation [29] reaching levels at term observed only in the hypothalamic portal system (median eminence) during physiological stress [25]. Abnormally accelerating rates or excessive levels of placental CRH are significant risk factors for an earlier onset of spontaneous birth [17,18,29,30,32,45,46]. Because of this, CRH is proposed to regulate a placental clock that controls a cascade of physiological events leading to parturition [42]. Despite general agreement concerning the significance of CRH for the timing of spontaneous birth [14], there is uncertainty about when CRH exerts its effects on human parturition [9,18,29] and what regulates the CRH surge [4].

CRH, a 41-amino acid neuropeptide, is synthesized primarily in the paraventricular nucleus of the hypothalamus and has a major role in regulating pituitary-adrenal function and the physiological response to stress [5,43]. During pregnancy, CRH also is synthesized by the placenta. Placental CRH is identical to hypothalamic CRH in structure, immunoreactivity and bioactivity [36,39]. In contrast, however, to the inhibitory influence on the promoter region of the CRH gene in the hypothalamus, maternal stress signals (glucocorticoids) from the adrenal glands activate the promoter region in the placenta and stimulate its synthesis [21,41]. This positive feedback system contains both a signal to the fetus (elevated glucocorticoids) that the host environment (the mother) is threatened [44], potentially compromising fetal survival, and a response from the fetus (increased placental CRH production) that shortens gestation. The purposes of our study are to determine the critical intervals during which CRH influences the length of human gestation and the critical periods during gestation when the placenta is most vulnerable to the biological signals of threat.

1. Methods

1.1. Participants

The sample was comprised of 203 English-speaking adult women (over 18 years age, mean age = 29.9 years) consecutively recruited from two University prenatal care programs between the 6th and 10th weeks of gestation. As presented in Table 1, the majority of the sample was married and high school educated. The sample was racially/ethnically diverse with a small majority that is white/non-hispanic. All eligible subjects presented with a singleton intrauterine pregnancy, a normal uterus and cervix. For the majority the current pregnancy was their first. A comprehensive, structured medical interview and thorough chart review was conducted to exclude subjects if they presented with prior or present obstetric risk conditions including systemic maternal disease (cancer, cardiac disease, seizure history, autoimmune diseases and blood disorders), placental or cord abnormalities, uterine anomalies, infection, congenital malformations or chromosomal abnormalities determined in the first trimester.

Table 1 – Subject characteristics (N = 203)

Age (years)	29.9 ± 5.25
Marital status (%)	Married: 78
Education (%)	High school: 99.5 Some college or college degree: 49.7
Race/ethnicity (%)	White/non-hispanic: 54.4
Parity (%)	Primiparous: 58.7
Gestational intervals (weeks)	Time 1: 15.3 ± 1.02 Time 2: 19.2 ± .75 Time 3: 24.9 ± .88 Time 4: 30.9 ± .97
Gestational age at term (weeks)	39.1 ± 1.71
Infant birth weight (g)	3446.24 ± 565.14

Data are presented as mean ± standard deviation.

Women also were excluded if they presented with any condition that could disregulate neuroendocrine function, such as endocrine, hepatic or renal disorders or the use of corticosteroid medications. Interviews assessed health behaviors to exclude women who smoked or consumed alcohol or drugs of abuse 6 months before and during the index pregnancy.

1.2. Procedures

All methods and procedures in this report were approved by the Institutional Review Board of the participating institutions. Women provided informed consent to be evaluated at four intervals during gestation; 13.5–16.6 (mean = 15.3), 17.8–20.5 (mean = 19.2), 23.7–26.5 (mean = 24.9) and 29.9–32.3 (mean = 30.9) weeks gestation. A clinical ultrasound performed at the first and second intervals confirmed gestational age. Blood was collected at each interval for assessment of neuroendocrine profiles. Women were followed to term and birth outcome information was abstracted from medical charts.

The HPA and placental stress axis was evaluated by assessing levels of B-endorphin (BE), ACTH, cortisol and CRH. To control possible circadian influences, subjects were evaluated each session between 14:00 and 16:00. The times for blood draws across sessions were not significantly different ($F_{3,200} = 1.14$, $p = .34$) and mean times ranged from 14:17 to 14:32. A 25 ml blood sample was withdrawn through antecubital venipuncture (within 20 s of venipuncture). Blood was deposited into siliconized and chilled EDTA (purple top) vacutainers, centrifuged at $2000 \times g$ (15 min) and the plasma was decanted into polypropylene tubes containing 500 KIU/ml aprotinin (to arrest enzymatic degradation; Sigma Chemical) and stored at -70°C until assayed.

CRH concentrations (pg/ml) were determined by radioimmunoassay (RIA; Bachem Peninsula Laboratories, San Carlos, CA). Plasma samples (1–2 ml) were extracted with three volumes of ice-cold methanol, mixed, allowed to stand for 10 min at 4°C , and then centrifuged at $1700 \times g$ for 20 min at 4°C by the modified method of Linton et al. [24]. The pellets were washed with 0.5 ml methanol, and the combined supernatants dried down (Savant SpeedVac concentrator).

Reconstituted samples in assay buffer were incubated with anti-CRH serum (human) for 48 h at 4 °C followed by a 24 h incubation with ^{125}I -CRH. Both labeled and unlabeled CRH were collected by immunoprecipitation with goat anti-rabbit IgG serum and normal rabbit serum after 90 min of incubation at room temperature. Samples were centrifuged at $1700 \times g$ (20 min) at 4 °C and the aspirated pellets were quantified with a gamma scintillation counter. The CRH assay had less than 0.01% cross-reactivity with ovine CRH, 36% cross-reactivity with bovine CRH and non-detectable reactivity with human ACTH. The intra- and inter-assay coefficient of variance ranged from 5 to 15%, respectively.

Plasma levels of adrenocorticotropin hormone (ACTH) were measured by RIA (Nichols Institute Diagnostics). The antiserum employed was <0.001% cross-reactive with beta-endorphin and ACTH fragments. Duplicate samples (200 μl /assay tube) were incubated with ACTH ^{125}I -antibody solution and an avidin coated bead overnight at room temperature. After washing, the bead with bound radiolabeled antibody complex was quantified using an ICN Biomedical Isoflex Gamma Counter. The ACTH assay has a minimal detectable dose (MDD) of 1.0 pg/ml (95% confidence) with intra-assay CV of 3.0% at 35 pg/ml and CV = 7.8% (inter-assay) at 36 pg/ml.

Plasma levels of BE were determined by a solid phase two-site immunoradiometric assay (IRMA; Nichols Institute Diagnostics). The antiserum was 1.6% cross-reactive with beta-lipotropin at 500 pg/ml and was <0.01% cross-reactive with related opiates at 5 $\mu\text{g}/\text{ml}$. The BE immunoassay system has a MDD = 14 pg/ml (95% confidence limit) with a CV = 4.1% (intra-assay) and CV = 9.0% (inter-assay) at the highest concentrations measured in the present study.

Plasma cortisol levels were determined by immunofluorescence using an automated procedure on an Abbott TDx Analyzer (Abbott Laboratories). The assay was less than 5% cross-reactive with 11-deoxycortisol, corticosterone, and less than 1% cross-reactive with ten other naturally occurring steroids. The inter- and intra-assay CVs were less than 9% with a minimum detectable level (95% confidence) of 0.45 $\mu\text{g}/\text{dl}$.

Data reduction for the RIA and IRMA assays were accomplished with a computer assisted, four-parameter logistics program [38]. Differences and rates of change in endocrine profiles between women delivering term and preterm were assessed with analysis of variance with fixed (Groups [term-preterm]) and repeated (assessment intervals-time) factors. Post hoc comparisons were computed using the Tukey method. Analysis of covariance was used to control for potentially confounding variables. Greenhouse-Geisser corrections were applied when appropriate and only corrected results are reported. Logistical multivariate regression was used to predict the dichotomous variable of preterm birth. General linear regression models were used to predict the separate continuous outcomes of gestational length and CRH levels at 31 weeks. Similar analytical strategies are used for both dichotomous and continuous variables. Initially, predictors (Table 1) were entered into the regression equation one variable at a time in no preset order but with the restriction that each entry increased the ability (the variance accounted for) to predict the separate outcomes of interest (conditional forward logical regression for preterm birth, and forward

stepwise regression for gestational length and CRH levels at 31 weeks). This approach was appropriate because the variables selected as predictors for this analysis were: (i) a cohesive collection of endocrine measures related to the stress axis and (ii) occurred in time before the outcomes of interest [16]. The stepwise solution was followed and confirmed by simultaneous regression, which considered concurrently all predictor variables, and then by hierarchical regression in which the order of predictor variables to enter the equation was selected by the investigator to control the influence of competing variables. The purpose of hierarchical regression was to determine causal priorities and to reduce confounding associations [6].

2. Results

2.1. Prediction of preterm birth

Placental CRH increased significantly ($F_{3,210} = 106.48$, $p < .0001$, Greenhouse-Geisser correction) during pregnancy with rapidly accelerating levels after 25 weeks gestation (Fig. 1, all pairwise comparisons $p < .0001$). Consistent with previous studies [29,45,46], placental CRH levels in women destined to deliver preterm (before 37 weeks) had faster rates of increase ($F_{3,603} = 5.73$, $p < .001$ [group \times weeks gestation]) and significantly higher levels of CRH confined to the beginning of the early third trimester ($F_{1,201} = 5.53$, $p = .02$ [post hoc comparison at 31 weeks]) than women who subsequently delivered at term (Fig. 2).

Maternal levels of cortisol, ACTH and BE also increased significantly with advancing gestation (all p 's < .0001). The two-fold increases in maternal ACTH and BE and the three-fold increase in maternal cortisol were considerably less than the 25-fold increases in placental CRH through 31 weeks of gestation (Table 2). Of these maternal measures, only cortisol distinguished women delivering term and preterm. This is the first evidence that levels of cortisol are higher as early as 15

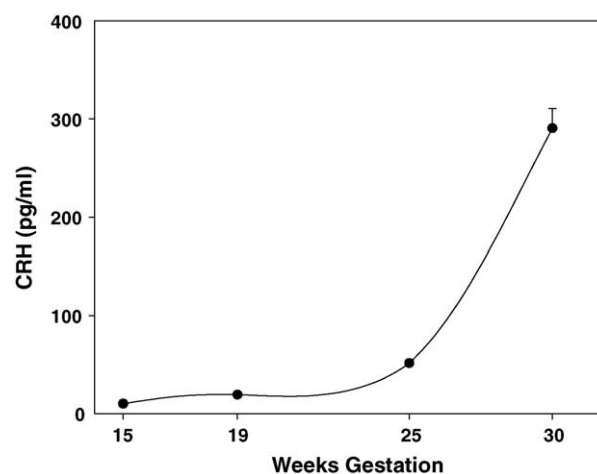


Fig. 1 – During the course of human pregnancy, levels of CRH increase as gestation progresses. All comparisons of CRH levels between time intervals are statistically significant.

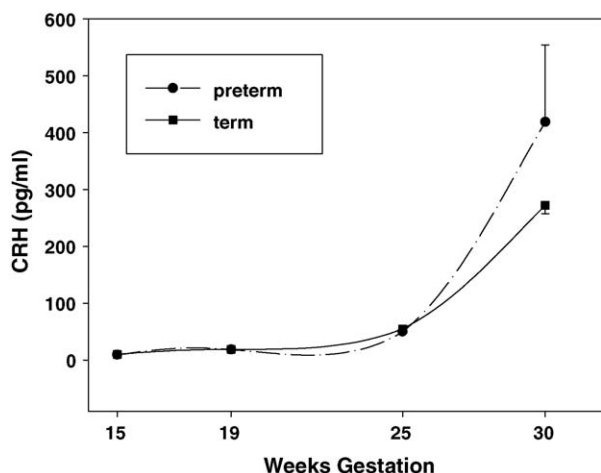


Fig. 2 - The rate of change in placental CRH between 26 and 31 weeks gestation is significantly faster and the level of placental CRH at 31 weeks gestation is significantly higher in women destined to deliver preterm.

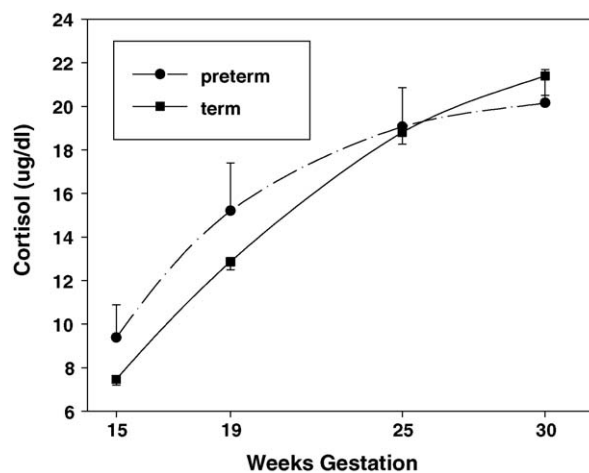


Fig. 3 - The level of maternal cortisol is significantly higher at 15 weeks gestation in women who will proceed to deliver before 37 weeks gestation (preterm).

weeks gestation ($F_{1,201}Y=4.45, p = .03$) with a similar trend at 19 weeks gestation ($F_{1,201}Y=3.43, p = .065$) in women who subsequently delivered preterm compared with women delivering after 37 weeks (Fig. 3).

Because levels of CRH late in pregnancy and concentrations of cortisol early in pregnancy both were associated with preterm birth, the association between CRH and birth outcome was re-evaluated after controlling for the level of cortisol. The rate of change of CRH during gestation ($F_{3,597} = 1.54, p = .19$) and the relation between CRH and preterm birth ($F_{3,597} = 2.39, p = .07$) were not significant when the statistical influence of cortisol at 15 weeks was controlled by analysis of covariance. However, when the independent contribution to the prediction of preterm birth was assessed using all of the endocrine variables (Table 2), only CRH at 31 weeks was selected by conditional logistic regression ($X^2_{d.f.=1} = 3.62, p = .05$).

Table 2 - Means and standard deviations for the endocrine stress variables and medical risk (N = 201)

Intervals weeks gestation (range)	Variables	Mean	Standard error
<i>Predictors</i>			
13.5-16.6 weeks	CRH	10.1 pg/ μ l	0.3
	ACTH	17.8 pg/ μ l	0.5
	BE	23.1 pg/ μ l	0.5
	Cortisol	7.7 μ g/dl	0.3
17.8-20.5 weeks	CRH	18.6 pg/ μ l	1.0
	ACTH	22.0 pg/ μ l	0.8
	BE	25.8 pg/ μ l	0.5
	Cortisol	13.1 μ g/dl	0.4
23.7-26.5 weeks	CRH	54.8 pg/ μ l	3.3
	ACTH	29.1 pg/ μ l	0.9
	BE	31.8 pg/ μ l	0.7
	Cortisol	18.8 μ g/dl	0.5
<i>Outcome</i>			
29.9-32.3 weeks	CRH	290.52 pg/ μ l	20.1

2.2. Prediction of gestational length

Similarly, when all variables were entered into the equation simultaneously, only placental CRH at 31 weeks significantly and independently predicted gestational length ($R = .20, p = .02$). The crucial role of CRH at 31 weeks for predicting gestational age at term was confirmed with Stepwise Regression. Elevated CRH at 31 weeks significantly (Step 1, $R = .20, F_{1,199}change = 8.36, p = .004$) predicted gestational length. The prediction of gestational length was improved by including in the model elevated cortisol at 15 weeks (Step 2, $R = .26, F_{1,197}change = 5.38, p = .02$), and finally elevated cortisol at 19 weeks (Step 3, $R = .29, F_{1,196}change = 4.08, p = .04$). Because levels of cortisol early in pregnancy, and levels of CRH late in pregnancy both resulted in the best prediction of gestational age at term, a final hierarchical model was constructed to control the early influence of cortisol in the equation. After cortisol levels at 15 and 19 weeks are entered in the equation (together at Step 1), elevation of CRH at 31 weeks still significantly predicted gestational age at term (Step 2, $R = .21, F_{1,197}change = 5.06, p = .02$).

2.3. Association between cortisol and CRH

The association between levels of cortisol and CRH across all time intervals is presented in Table 3. The purpose of this analysis was to examine all possible lagged and concurrent relations of these two hormones. There were no concurrent relations between cortisol and CRH for any time interval. Only the lagged associations of cortisol at 15 weeks ($R = .49, p < .001$) and 19 weeks ($R = .28, p < .01$) with CRH at 31 weeks were statistically significant.

2.4. Prediction of third trimester CRH Levels

Because CRH level at 31 weeks was the best predictor both of preterm birth and gestational length, a model to predict its

Table 3 – Correlations between cortisol and CRH at four gestational intervals (N = 203)

	T ₁ CORT	T ₂ CORT	T ₃ CORT	T ₄ CORT
T ₁ CRH	.041			
T ₂ CRH	.062	.098		
T ₃ CRH	.058	.073	-.010	
T ₄ CRH	.487**	.277**	.078	-.026

T₁ = 15 weeks, T₂ = 19 weeks, T₃ = 25 weeks and T₄ = 31 weeks.
** p < .01.

precipitous rise during gestation was constructed using all endocrine stress markers collected from the beginning of the second trimester (15 weeks) through week 26 of gestation (Table 2). The prediction of CRH levels at 31 weeks using all the endocrine markers was highly significant ($R = .58$, $d.f. = 12,188$, $F_{\text{change}} = 8.04$, $p < .0001$). Stepwise regression revealed that the single best and highly significant ($R = .49$, $d.f. = 1,199$, $F_{\text{change}} = 61.78$, $p < .0001$) independent predictor of third trimester CRH was cortisol at 15 weeks gestation. Every unit of change ($\mu\text{g/dl}$) in cortisol at 15 weeks was associated with a change in 34 units (pg/ml) of CRH at 31 weeks. The significant association between early levels of cortisol and concentrations of CRH at 31 weeks remained unchanged when analyzed by methods that controlled for the statistical influence of all other endocrine variables measured.

A final stepwise model was constructed with three variables that accounted for the most variance in the prediction of third trimester CRH (Table 4). In addition to elevated maternal cortisol at 15 weeks (Step 1), the model included elevated CRH at 26 weeks (Step 2) and elevated maternal cortisol at 19 weeks (Step 3). After the initial contribution of cortisol at 15 weeks, each of the two additional variables resulted in a progressive and significant increase in the ability to predict CRH at 31 weeks; however, the magnitude of these subsequent contributions was very small, ranging between 2 and 5% of the variance (Table 4).

3. Discussion

Our longitudinal study of human pregnancy provides convincing evidence that the earliest, and perhaps critical, period for the effects of CRH on gestational length is restricted to the interval between weeks 26 and 31. The rate of increase during this interval is faster and the level of CRH at 31 weeks is higher in women destined to deliver preterm. Serial sampling of maternal plasma provides no support for the possibility that CRH earlier in pregnancy influenced gestational length. These results confirm that elevated CRH after 26 weeks, but not before, constitute a significant risk for preterm birth [18]. New

findings from this study indicate that a plausible stress-related endocrine signal, elevated cortisol [22,37] from the mother very early in pregnancy predicts the precocious rise in CRH leading to an abbreviated gestational period.

Previous research indicated that exposure to stress, especially early in pregnancy, may accelerate the rise in placental CRH and shorten the length of gestation [13]. The pattern of findings in the current study supports the argument that the effect of elevated cortisol early in pregnancy on gestational length reflects a priming or programming [2] effect on the eventual fetal/placental CRH response. First, elevation in maternal cortisol, a primary endocrine response to stress [22,37] during the first 15 weeks of gestation significantly predicts the length of gestation. This finding is consistent with the recent report of decreased gestational length among women administered corticosteroids during their first trimester [15]. Second, the relation between early elevations of cortisol and length of gestation is associated with increased levels of CRH later in pregnancy. Third, there is no effect on gestation of other maternal stress signals (ACTH or BE) at any time interval. Cortisol early in gestation is the only stress-related signal that predicts both CRH levels at 31 weeks and gestational length. Fourth, our findings in vivo of a positive association between cortisol and placental CRH are consistent with in vitro studies describing activation by glucocorticoids of placental CRH synthesis [21,41]. Furthermore, administration of synthetic glucocorticoid (betamethasone or dexamethasone) to pregnant women at risk for preterm delivery (to mature the fetal lungs) is associated with significant increases in circulating maternal CRH [28]. These findings unequivocally support the ability of cortisol to stimulate in vivo placental CRH; however, the stimulation of CRH only was seen in women after 30 weeks gestation and within 3 h of administration of the synthetic compound.

The time course for the effect of synthetic glucocorticoid on placental CRH is not consistent with the early (15 weeks gestation) and lagged (16 weeks later) association between maternal cortisol and CRH elevation observed in the present study. There are at least two explanations for these differences. First, synthetic glucocorticoid is administered in doses that profoundly alter the HPA axis [28] and reflect pharmacological rather than physiological effects. Second, an enzyme in the placenta, 11B-HSD2, oxidizes physiological (maternal) but not synthetic glucocorticoids into cortisone [19,21]. This protects the fetus from the direct and sometimes harmful effects of exposure to maternal cortisol during critical periods of development [47]. The levels of placental 11B-HSD2 rise as gestation progresses before abruptly falling near term ensuring fetal organ maturation in full term births [26,31]. It is possible that an immature placental defense system early in gestation is not capable of converting excessive levels of maternal cortisol. Early exposure to

Table 4 – Final model from stepwise multiple regression for the prediction of third trimester CRH

Steps	Variable	Standardized beta	Unstandardized beta	R	R ² change	F _{change}	P-value
Step 1	Cortisol (15 weeks)	.49	33.5	.49	.24	61.70	.000
Step 2	CRH (25 weeks)	.23	1.4	.54	.05	14.61	.000
Step 3	Cortisol (19 weeks)	.16	7.7	.56	.03	6.4	.012

cortisol may prime the placental clock [30,42] and accelerate placental synthesis and release of CRH [21,41]. By the same reasoning, the effects of elevated maternal cortisol at 31 weeks does not result in a simultaneous increase of maternal CRH because the activity of placental 11 β -HSD2 is sufficient to convert the cortisol to cortisone. Interestingly, and in agreement with an earlier study [27], cortisol and CRH are significantly and concurrently associated only at 37 weeks in a subset of women ($N = 164$) in our sample consistent with the timing of the abrupt fall in 11 β -HSD2. The findings suggest that a profile of endocrine markers, led primarily by biological evidence of maternal stress early in pregnancy, program a subsequent fetal/placental response that results in foreshortened gestation.

Early detection by the fetal/placental unit of stress signals from the maternal environment "informs" the fetus that there is a threat to survival. This information primes or advances the placental clock by activating the promoter region of the CRH gene and increases the synthesis of the gene product. The rapid increase in CRH begins the cascade of events resulting in myometrial activation and fetal escape from a malignant environment. Early departure from the inhospitable host environment may be essential for survival but it does have grave consequences for the tadpole and the human fetus. The immature tadpole is at a disadvantage competing with normally developing tadpoles foraging for food and reproducing [12]. Human infants born early suffer a similar fate that includes a panoply of motor, sensory and neurological impairments that persist for a lifetime [1,33].

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