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Sexually dimorphic adaptations in basal maternal stress physiology during pregnancy and implications for fetal development

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1.0 Introduction

Human pregnancy induces alterations to maternal stress physiology that appear, at least in part, to be driven by signals derived from the fetal-placental unit (Clifton et al., 2012; Dipietro et al., 2004; Glynn, 2010; Glynn and Sandman, 2011). The placenta has both sensory and effector functions that facilitate two-way communication between the fetus and mother. The fetus makes developmental adaptations based upon signals from the maternal environment and the fetal-placental unit promotes adaptation within maternal cardiovascular, respiratory and endocrine systems to meet the demand for resources essential to fetal development (Wadhwa et al., 2002). Despite evidence of reciprocal exchange of information between the mother and fetus (Glynn, 2010; Glynn and Sandman, 2011; Sandman et al., 2012), the majority of research has focussed on the *fetus* as a recipient of signals from the mother. Specifically, there is an emerging consensus across animal and human studies of sexually dimorphic fetal-placental adaptations to signals emanating from the maternal environment (Bale, 2011; Clifton, 2010; Clifton et al., 2012; Sandman et al., 2013). For example, maternal psychological distress during human gestation is associated with relatively greater risk for neurodevelopmental impairments in male offspring and for affective problems in female offspring (Davis and Pfaff, 2014; Sandman et al., 2013). Little attention has been given to the *mother* as recipient of signals from the fetus or to the implications of sex differences in maternal adaptation for fetal development. In the current study we focus on the contributions of fetal sex to maternal adaptations within two important components of maternal stress physiology: the hypothalamic-pituitary-adrenal (HPA) axis and the autonomic nervous system (ANS).

1.1. Changes in maternal stress physiology during pregnancy

During pregnancy, maternal stress response systems undergo major changes to support the combined maternal and fetal needs for energy. The ANS initiates a shift toward greater sympathetic and reduced parasympathetic control (DiPietro et al., 2005) accompanied by increases in blood volume and heart rate as well as a reduction in systemic vascular resistance to bring about the increase in cardiac output necessary to sustain pregnancy (Silversides and Colman, 2007). Maternal-fetal communication via ANS pathways is made more complex by dampening of ANS mediated responses to stress during pregnancy (de Weerth and Buitelaar, 2005; Entringer et al., 2010; Klinkenberg et al., 2009; Matthews and Rodin, 1992; Nierop et al., 2006). For example, Nierop and colleagues reported that increases in salivary alpha-amylase (sAA) following a psychosocial stressor were markedly attenuated in pregnancy compared to nonpregnant women (Nierop et al., 2006). Although attenuated, sAA remains a sensitive biomarker for emotional arousal during pregnancy (Giesbrecht et al., 2013b). sAA has a diurnal pattern that is opposite to cortisol; waking levels are modest with a sharp decrease in the first hour post waking and then increases over the remainder of the day (Nater and Rohleder, 2009), a pattern which is preserved during pregnancy (de Weerth and Buitelaar, 2005). Despite evidence of sex-specific maternal adaptations within ANS function during pregnancy (see below), differences in basal sAA during pregnancy as a function of fetal sex have not been studied.

Pregnancy also initiates changes within the maternal HPA axis. Cortisol levels increase dramatically, peaking in the third trimester at three times non-pregnancy levels (Jung et al., 2011). Rises in basal cortisol during pregnancy are due, in part, to the stimulatory effect of placental corticotropin-releasing hormone on the maternal pituitary and adrenal glands (Sandman et al., 2006). Cortisol, the end-product of the HPA axis, reliably increases following exposure to mental stress, even during pregnancy (Giesbrecht et al., 2013a; Giesbrecht et al., 2012). Despite

dramatic increases in maternal cortisol during pregnancy, the normal diurnal pattern of cortisol (modest levels upon awakening followed by a sharp increase within the first hour after waking and then decreases over the course of the day) remains unchanged (Entringer et al., 2011).

It is widely reported that HPA axis reactivity to stressors is progressively attenuated with advancing gestation. Indeed, there is clear evidence of such attenuation within the animal (Neumann et al., 1998; Russell et al., 2008; Slattery and Neumann, 2008) and human (Kammerer et al., 2002; Odagiri et al., 1988; Schulte et al., 1990) literature with regard to chemical and physical stimulation of the HPA axis. In contrast, studies involving social and mental stressors generally report robust HPA axis responses throughout pregnancy (De Weerth et al., 2007; Giesbrecht et al., 2013a; Nierop et al., 2006) although some studies have not (Entringer et al., 2010; Evans et al., 2008). In the Entringer study, accommodations designed to reduce physical discomfort for pregnant women likely reduced the overall potency of the stressor. We have argued that the overall pattern of findings suggests a selective dampening of the HPA axis during pregnancy (Giesbrecht et al., 2013a), which may result from differences in activation of neural pathways used to process psychological stimuli (e.g., rostral cortico-limbic structures) compared to those involved in processing physical or chemical stimulation (e.g., primitive caudal brain regions) (Russell et al., 2008). Nevertheless, in the present study we have included gestational age and psychosocial distress as covariates to account for the possibility of pregnancy-related changes in the responsivity of the ANS and HPA axis to stress during pregnancy.

1.2. Effects of fetal sex on maternal physiology during pregnancy

Some of the substantial alterations of maternal physiology during pregnancy are sex-specific. Fetal sex influences maternal glycemic control and blood pressure, especially among women genetically susceptible to metabolic and cardiovascular disease (Hochoer et al., 2009;

Petry et al., 2007). Maternal vascular function is influenced by fetal sex in both normal and pre-eclamptic pregnancies but the direction of the effect differs as a function of pregnancy complication. In normal pregnancy, women carrying a male fetus tend to have greater peripheral *vasodilation* relative to women carrying a female fetus whereas in pregnancies complicated by pre-eclampsia, women carrying a male fetus have greater *vasoconstriction* relative to women carrying a female fetus (Stark et al., 2006). The reasons for this difference are not known, but sexually dimorphic fetal growth strategies may play a role via the male fetus attempting to maximize growth by redirecting maternal blood flow from peripheral to the utero-placental circulation. In pregnancies complicated by asthma, asthma severity is exacerbated more often in asthmatic women carrying a female fetus (Clifton, 2010; Clifton and Murphy, 2004; Clifton et al., 2012), and treatment of asthma with inhaled glucocorticoids (which normally is associated with adrenal suppression) results in cortisol inhibition only in women carrying a female fetus (Hodyl et al 2011). Again, the specific mechanisms are not known but it is believed that sex-specific placental cytokine expression and response to cortisol inhibition, may contribute to the sex-specific course of maternal asthma during pregnancy. These examples illustrate the importance of fetal sex to the regulation of maternal physiology during pregnancy.

DiPietro and colleagues (DiPietro et al., 2011) reported that pregnancy-related adaptations within the maternal HPA axis in normal pregnancy differ by fetal sex. Mean levels of maternal cortisol were lower in the second trimester among women carrying a female fetus compared to women carrying a male fetus, but in the third trimester the pattern was reversed. These findings are intriguing because cortisol plays an important role in fetal growth and lung maturation, both of which differ by fetal sex. Investigating whether fetal sex is associated with patterns of diurnal secretion has the potential to yield new insight into the effects of fetal sex on regulation of

maternal stress physiology during pregnancy and how such regulation may feed back to the fetus to regulate its growth.

1.3. Associations between maternal cortisol and offspring birth weight

It has long been known that psychological distress during pregnancy is associated with increased risk for adverse birth outcomes (Alder et al., 2007; Berkowitz et al., 2003) and, at least among depressed women, cortisol is one of the mechanisms responsible for this association (Diego et al., 2009). Cortisol is known to be an important factor in fetal growth. Elevated levels of cortisol during normal pregnancy are associated with reduced fetal size and fetal growth rate at midgestation (Diego et al., 2009; Diego et al., 2006) and reduced birth weight (Bolten et al., 2011; Diego et al., 2009; Ghaemmaghami et al., 2014; Kivlighan et al., 2008), although several large studies found this association was no longer significant after including relevant covariates, such as gestational age at birth (Goedhart et al., 2010; Li et al., 2012). Increased levels of placental corticotropin-releasing hormone are also associated with reduced fetal growth (Wadhwa et al., 2004). In pregnancies complicated by maternal asthma, female but not male birth weight percentiles were inversely associated with cord blood cortisol levels (Hodl et al., 2010). Together, these findings point toward an important role for the maternal HPA axis in regulating fetal growth.

To the extent that diurnal patterns of maternal stress physiology differ by fetal sex, these differences may have relevance to sexually dimorphic fetal growth strategies (Davis and Pfaff, 2014; Sandman et al., 2013). Males invest resources in growth, a strategy that likely contributes to their larger size at birth compared to females but increases their vulnerability to subsequent intrauterine adversity as observed in higher rates of intrauterine growth restriction, preterm birth and stillbirth among males (Clifton et al., 2010; Hodl et al., 2010). In contrast, females conserve

resources and adjust their growth to match maternal conditions – a strategy that increases their ability to adapt under conditions of adversity (Clifton, 2010). Because of their direct and substantial role in regulating the availability of energetic resources for both maternal and fetal needs, the maternal stress response systems represent plausible pathways that contribute to the regulation of fetal growth.

1.4. Present study

Our aim was to determine whether diurnal patterns of maternal stress-related physiology during pregnancy differ by fetal sex and growth. The specific objectives of the present study were to determine whether fetal sex is associated with diurnal fluctuations in basal ANS and HPA axis function (as indexed by sAA and salivary cortisol, respectively) in pregnant women and determine whether sAA and cortisol are associated with fetal growth. We reasoned that if fetal sex contributes to adaptations with maternal stress physiology then it should moderate diurnal patterns of sAA and cortisol. Furthermore, if sex-differences in maternal sAA and cortisol are related to sexually dimorphic fetal growth strategies, then sAA and cortisol should be related to birth weight. To test these questions, we collected diurnal saliva samples during pregnancy and modeled basal diurnal growth trajectories for sAA and cortisol as a function of fetal sex and birth weight.

2.0. Methods

2.1. Participants

Two hundred ninety five women were recruited prior to 22 weeks of gestation. Four women subsequently miscarried and were not included in these analyses. The sample was drawn from the Alberta Pregnancy Outcomes and Nutrition study (www.apronstudy.ca), a prospective longitudinal study that is following a community sample of healthy volunteers. Women were

excluded if they smoked or consumed alcohol during pregnancy, had a non-singleton pregnancy or were receiving synthetic glucocorticoids for the treatment of asthma or atopic dermatitis. The sample was relatively well-educated (89.7 % had education beyond high school), non-Hispanic White (77.3%), financially stable (mean household income \$70,000-\$99,999 CAD/annum), married or common-law (94.9 %), mature (mean age = 31.4 years, SD=3.8) and primipariopus (50%). Obstetric risk conditions were present in 48 (16.5%) pregnancies of which 22 had preeclampsia, 11 had gestational diabetes, 18 infants were born preterm, and 8 infants were born small for gestational age. Some fetuses were exposed to multiple adversities. The sex ratio favored boys ($n = 151$; 51.9%), consistent with the worldwide birth ratio of 51.7% males and 48.3% females (Hesketh and Xing, 2006).

2.2. Procedure

Participants collected diurnal suites of saliva at two points in pregnancy: T1 = on two days prior to 22 weeks gestation ($M = 14.9$, $SD = 3.7$), and T2 = two days at 32 weeks gestation ($M = 32.4$, $SD = 1.0$). At each time point, participants self-collected saliva at home during regular weekdays on the following schedule supported by a Personal Digital Assistant (PDA, i.e. PalmTM): upon waking, 30 minutes after waking, at 1130h, and at 2100h. On each sampling day, participants collected the waking sample as soon after waking as practically possible and they initiated a 30 minute timer on the PDA. This procedure allowed for precise timing of the waking plus 30 minute sample while also allowing for individual waking times. With the exception of the waking sample, the PDA rang to indicate that a sample was to be collected.

Each time the PDA rang, it provided a unique code corresponding to a pre-labeled saliva tube and instructed the participant to place the saliva roll under her tongue. The time of each assessment was recorded by the PDA, permitting precise modeling of diurnal patterns. To

facilitate adherence to the study protocol, the PDA allowed a 20 minute response window following the signal, after which data were considered missing.

2.3. Salivary biomarkers

Participants were asked to refrain from consuming food, caffeine, citric drinks and dairy, to avoid vigorous exercise or brushing teeth in the 30 minutes prior to saliva sample collection, and to report adherence to these guidelines. Saliva samples were temporarily stored in participant home freezers (usually only 1 day) until they could be transported frozen to the laboratory where they were stored at -80 C until shipped frozen to Salimetrics (State College, PA). All samples were assayed for sAA and salivary cortisol without modification to the manufacturer's instructions.

The sAA assay employs a chromagenic substrate, 2-chloro-p-nitrophenol linked to maltotriose. The enzymatic action of sAA on this substrate yields 2-chloro-p-nitrophenol, which can be spectrophotometrically measured at 405 nm using a standard laboratory plate reader. The amount of sAA activity present in the sample is directly proportional to the increase (over a 2 minute period) in absorbance at 405 nm. Results were computed in U/mL of sAA. The assay requires 10 μ L of saliva. Intra-assay coefficient of variation was 5.5%. Inter-assay variation was 4.7%.

The Salimetrics cortisol test is a 510k cleared high-sensitivity enzyme immunoassay specifically designed for use with saliva. The assay requires 25 μ L, has a lower limit of sensitivity at 0.007 μ g/dL, standard curve range from 0.012 to 3.0 μ g/dL, and average intra-and inter-assay coefficients of variation 3.5% and 5.1% respectively. Method accuracy, determined by spike and recovery, and linearity, determined by serial dilution are 100.8% and 91.7%. In the current study, a random 10% of samples were assayed in duplicate to confirm reliability; the

intra-assay coefficient of variation was 3.5%. The mean value from duplicate samples was used for data analysis.

2.4. Obstetric risk conditions

Indicators of obstetric risk conditions were determined by medical chart review following delivery. We included obstetric risk conditions that involve components of mental stress response systems in their underlying pathophysiology, including asthma, pre-eclampsia, gestational diabetes, preterm birth and small for gestational age. Asthma diagnosis was determined by maternal self-report and confirmed by chart review. Preeclampsia and gestational diabetes were defined using the Society of Obstetricians and Gynaecologists of Canada guidelines (Berger et al., 2002; Magee et al., 2008). Preterm birth was defined as delivery prior to 37 weeks gestation. Gestational age was determined by a first trimester ultrasound. Small for gestational age was defined as the 5th percentile or lower.

2.5. Psychological distress

Psychological distress was assessed at each time point in pregnancy using reliable and valid measures of depression and anxiety. To achieve an overall measure of psychological distress, the individual measures were first standardized using a z-score transformation and then combined to create an overall estimate for each individual.

Depression: The *Edinburgh Depression Scale* is a 10-item self-report instrument that has been used extensively and is shown to be a reliable, valid, and sensitive measure of depression symptoms among pregnant women (Cox et al., 1987; Jomeen and Martin, 2007). Scores range between 0 and 30 with scores greater than 12 strongly associated with physician diagnosis of Major Depressive Disorder (Cox et al., 1987). Sensitivity (100%) and specificity (87%) are also satisfactory when used with pregnant women (Murray and Cox, 1990).

Anxiety: The *Symptom Checklist-90-R* (Derogatis, 1994) is a multidimensional self-report symptom inventory; for present purpose we used only the 10-item anxiety scale. This anxiety scale has good convergent and divergent validity (Morgan et al., 1998) and adequately discriminates between clinical and non-clinical samples (Holi et al., 1998). Scores range from 0 to 40.

Pregnancy Specific Anxiety: The *Pregnancy Specific Anxiety Scale* (Rini et al., 1999) is a 10-item self-report instrument that has been used to assess the extent to which pregnant women worry about their health, their baby's health, labor and delivery, and caring for their baby. This reliable measure (Cronbach's $\alpha=.81$) was specifically developed for use in pregnancy research. Scores range from 0 to 30.

2.6. Birth weight percentile

Birth weight was extracted from the birth record and used to obtain birth weight percentiles, adjusted for sex and gestational age at birth, according to growth charts derived from the 1999-2000 US Natality Datasets (Oken et al., 2003).

2.7. Data analysis

Hierarchical linear models were estimated using HLM 7.1 (Raudenbush et al., 2011) to determine whether diurnal changes in maternal sAA and cortisol were associated with fetal sex and birth weight percentile. These models are ideal for analysis of nested data (individual saliva samples nested within the two timepoints and all nested within individuals). Because early morning and daytime changes in basal sAA and cortisol represent different aspects of ANS and HPA axis function (Fries et al., 2009; Nater et al., 2007), separate models were tested for the awakening response and the daytime slope. Models for the awakening response included the waking and waking plus 30 minute samples as outcome with time centered at each individual's

waking. Due to the dependence of the cortisol awakening response on sample timing, samples were excluded if they were taken more than 15 minutes after waking for the waking sample ($n = 180$; 15.3%) and more than 50 minutes after waking for the 2nd sample ($n = 119$; 9.9%) (Okun et al., 2010; Wust et al., 2000).

Models for the daytime slope included samples collected 30 minutes post waking, mid-morning, and evening. Daytime models parameterized time as time-since-waking (Time) and time-since-waking squared (Time²) to account for non-linear changes in daytime sAA and cortisol. Time was centered at the beginning of the daytime trajectory (30 min post waking); models centered at other time points (e.g., noon) yielded virtually identical results.

Hierarchical linear equations were specified at three levels to account for the nested data structure (saliva samples nested within the two timepoints and timepoints nested within persons). In the level 1 submodel the cortisol samples (as described above) were modeled as a function of time (to estimate their change over the day). The level 2 and level 3 submodels were the same across all models tested. In preliminary models we determined that the awakening response and the daytime slopes for both sAA and cortisol differ as a function of advancing gestation. Therefore, we included gestational age (centered at the sample mean to estimate the overall linear trend) in the level 2 submodels for the intercept and slopes. The primary variables of interest, fetal sex and birth weight percentiles, were included in the level 3 submodel. Males were the reference group for the sex variable and the results therefore report the specific effects for males and the differences between males and females. Birth weight percentiles were centered at the 50th percentile. All models were adjusted for maternal covariates that are known to influence stress physiology during pregnancy or have the potential to affect sex ratios (Jones et al., 2006), including maternal prenatal psychological distress, obstetric risk conditions, parity,

pre-pregnancy BMI, and age. Random effects allowed variation in salivary biomarkers at the intercept and the change over the course of the day. Cortisol and sAA were significantly skewed, even after removing samples greater than 4 SD from the mean ($n = 2$ for cortisol; $n = 26$ for sAA) and thus were log transformed prior to analyses. For ease of interpreting log transformed model coefficients are interpreted as the percentage change in outcome per unit change in the independent variable using the following transformation: $\beta_{\% \text{change}} = 100 * [\exp(\beta \ln)] - 1$.

3.0. Results

Descriptive data for study variables and sample characteristics are presented in Table 1. The final sample size was 291 at T1 and 259 at T2. There were no fetal sex differences ($p > .05$) for any of the covariates included in the mixed models.

3.1. Awakening responses

sAA. sAA levels decreased by 55.6% ($\beta = -.81$, $p < .001$) below waking levels within 30 minutes of awaking. Sex did not affect sAA waking levels, $\beta = -.085$, $p = .44$, or the sAA awakening response, $\beta = .021$, $p = .86$; complete model results are available in Table S1.

Cortisol. Cortisol levels increased by 64.9% ($\beta = .50$, $p < .001$) over waking levels within 30 minutes after waking. Sex did not affect waking cortisol levels ($\beta = -.032$, $p = .45$) or the cortisol awakening response ($\beta = -.058$, $p = .39$); see Table S1.

3.2. Daytime trajectories

sAA. The growth trajectory for sAA was a steep increase during the early part of the day ($\beta = .162$, $p < .001$) with a gradual flattening over the remainder of the day ($\beta = -.009$, $p < .001$); see Table S2 for complete model results. As shown in Figure 1, the daytime sAA growth trajectory differed by fetal sex. Relative to the growth trajectories for males, female sex resulted in a 4.1% increase per hour in the linear slope ($\beta = .040$, $p = .028$). Female sex was also

associated with a 0.2% decrease per hour² in the quadratic slope ($\beta = -.002$, $p = .030$). The overall daytime pattern displayed in Figure 1 indicates greater secretion of sAA in women carrying a female compared to male fetus.

Cortisol. The daytime growth trajectory for cortisol was a steep decline in cortisol during the early part of the day ($\beta = -.253$, $p < .001$ for the linear slope) with a gradual flattening over the remainder of the day ($\beta = .009$, $p < .001$ for the quadratic slope). Fetal sex moderated this trajectory. Relative to males, participants carrying a female fetus had significantly flatter linear ($\beta = .039$, $p = .003$) and quadratic ($\beta = -.002$, $p = .006$) slopes; see Table S2. As shown in Figure 2, flatter daytime growth trajectories for women with a female fetus resulted in greater overall daytime cortisol secretion relative to women carrying a male fetus.

3.3. Effects of advancing gestation

As expected, advancing gestation was associated with changes in the diurnal patterns for both sAA and cortisol (see Tables S1 and S2). Nevertheless, there were no interactions between gestational week and fetal sex, indicating that the effects of advancing gestation on the diurnal patterns did not differ as a function of sex (see Tables S1 and S2).

3.4. Associations with birth weight percentile

sAA awakening response. Birth weight percentile was associated with sAA levels at waking ($\beta = .003$, $p = .044$) and the decrease in sAA after waking ($\beta = -.004$, $p = .045$) such that higher waking sAA and a more robust decrease after waking were associated with higher birth weight percentiles; see Table S3.

Cortisol awakening response. Waking cortisol and the cortisol awakening response were not associated with birth weight, $\beta = .0004$, $p = .54$ and $-.001$, $p = .40$, respectively; see Table S3.

sAA daytime trajectory. The daytime sAA trajectory was not associated with birth weight percentile, all p 's $> .68$; see Table S4.

Cortisol daytime trajectory. The linear cortisol slope, $\beta = -.0002$, $p = .028$, but not the quadratic slope, $\beta = .000001$, $p = .97$, was associated with birth weight; see Table S4. As shown in Figure 3, women with flatter daytime trajectories gave birth to infants with lower birth weight.

Although the birth weight percentiles were adjusted for sex and gestational age at birth, we nevertheless tested models that included interactions between sex and birth weight percentile to determine whether sex moderated the effects of sAA and cortisol on birthweight. None of these interaction effects were significant (results not shown).

4.0. Discussion

This study demonstrated an association between fetal sex and diurnal patterns of maternal sAA and salivary cortisol during pregnancy. Women carrying a female fetus had flatter daytime cortisol patterns and elevated daytime sAA slopes compared to women with a male fetus. These patterns of diurnal function resulted in greater total sAA and cortisol secretion in women carrying a female fetus. Diurnal patterns of sAA and cortisol were also associated with birth weight percentiles such that women with flatter daytime cortisol and blunted sAA awakening response gave birth to lower birth weight infants. Overall, the pattern of results suggests that fetal sex plays a role in regulating maternal stress physiology during pregnancy. Furthermore, these observations are consistent with the proposal that sex differences in maternal sAA and cortisol are related to sexually dimorphic fetal growth strategies.

Within clinical populations, flattened daytime cortisol patterns have generally been interpreted as indication of HPA axis dysfunction (Miller et al., 2007). The few previous studies that have reported on daytime slopes in pregnant women suggest that flattening is associated

with psychological distress (Kivlighan et al., 2008; Obel et al., 2005). These studies have several implications for understanding the flattened cortisol patterns we observed in women carrying a female fetus. First, although there was no sex difference in psychological distress, flattened cortisol in participants with a female fetus is consistent with distress-related flattening of cortisol that results in reduced fetal growth. It has long been suspected, and Diego colleagues (Diego et al., 2009) have provided supporting evidence, that cortisol mediates the association between psychological distress and fetal growth. Our findings suggest that cortisol may be a general mechanism contributing to sex differences in fetal growth, even after accounting for the effects of psychological distress. This proposal is supported by several lines of research, including studies showing an association between increased maternal cortisol and reduced fetal growth in normal pregnancy (Bolten et al., 2011; Ghaemmaghami et al., 2014) and reductions in birth weight consequent to exogenous glucocorticoid treatment for threatened preterm birth (Murphy et al., 2006) and maternal asthma (Hodyl et al., 2010), although in the latter case the findings were only for females. The fact that sex did not moderate the association between birth weight and maternal cortisol again points to cortisol as a general mechanism regulating growth.

A second potential implication of our findings is that greater cortisol exposure among females may contribute to sex differences in the incidence and presentation of stress-related disorders (Weinstock, 2007). Greater prenatal cortisol exposure is associated with increased stress reactivity and sex-specific development of neurobiological pathways involved in stress regulation (Buss et al., 2011; Buss et al., 2012; Davis et al., 2011; Sandman et al., 2011; Werner et al., 2013), which may contribute to greater female risk for affective problems (Davis and Pfaff, 2014; Sandman et al., 2013).

Our findings regarding sex difference in maternal sAA during pregnancy are novel. Relatively higher diurnal sAA trajectories among women with a female fetus suggest relatively more autonomic arousal, which may contribute to sex differences in fetal growth strategies. The female fetus takes a ‘balanced’ approach to allocation of resources – it tends to limit growth, especially under conditions of adversity, in favor of maintaining resource reserves that can be allocated toward survival in the event of continued or worsened adversity (Clifton, 2010). In addition to ensuring availability of energetic resources for growth, relatively increased autonomic arousal may also increase maternal survival fitness within adverse environments through processes such as heightened vigilance. This proposal is consistent with the overall function of ANS but it is also consistent with the specific role of sAA to aid in harvesting calories from starches and carbohydrates.

Blunting of the sAA awakening response in association with reduced birth weight percentile is also a novel finding. We speculate that sAA blunting is an indicator of relatively poor ANS regulation and this in turn has an effect on fetal growth. This speculation is supported by the fact that secretion of sAA is sensitive to psychological distress, even during pregnancy (Giesbrecht et al., 2013b), and by the negative effect of psychological distress on fetal growth.

Taken together, the findings for sAA and cortisol suggest that fetal regulation of maternal stress physiology is consistent with sexually dimorphic fetal growth and maturation. By upregulating the maternal ANS and HPA axis, the female fetus helps to ensure a balance between growth and conservation of resources that enhances female adaptability and survival.

Our findings are partially consistent with and extend the work of DiPietro and colleagues (DiPietro et al., 2011) who reported that mean levels of cortisol during the second half of pregnancy are sex-dependant. In that study, single measures of afternoon salivary cortisol were

obtained at weekly intervals starting at mid gestation, which is an ideal design to assess sex-dependant changes in cortisol as a function of advancing gestation. Cortisol levels in women carrying a female fetus were lower relative to those with a male at mid gestation but higher throughout the third trimester. The current study was not designed to address changes as a function of advancing gestation but rather to determine whether *diurnal* patterns of cortisol are also sex-dependant. The findings for the third trimester in the DiPietro study are consistent with our proposal that maternal adaptations provide environmental support for sex-specific fetal growth strategies. The third trimester is a period of rapid fetal growth, and increasing concentrations of cortisol retard growth (Bolten et al., 2011; Goedhart et al., 2010; Reynolds, 2013); thus higher third trimester cortisol in women carrying a female fetus is consistent with the female strategy to limit growth and conserve resources as a means to reduce morbidity and mortality.

Given the correlational nature of our data, some questions remain unanswered. For example, we wonder to what extent our observations of sex-differences in maternal stress physiology can be attributed to equal but opposite effects of the male and female fetus or primarily to the effects of only males versus females. Male growth strategies have been characterized as relatively impervious to maternal signals whereas the female strategies incorporate a broad range of signals about maternal adversity accompanied by relatively large reductions in female growth (Clifton, 2010). To the extent that larger fetal adaptations correspond also to larger maternal adaptations, at least as far as sAA and cortisol are concerned, we could expect greater adjustments within maternal physiology when pregnant with a female compared to male fetus. Studies that assess maternal stress physiology prior to pregnancy are needed to determine the relative influence of male and female sex.

Another important limitation of our research is that we cannot rule out the possibility that the observed sex differences in maternal stress physiology *select* for sex and that sexually dimorphic maternal physiology is in fact the *cause* of fetal sex selection. The causes of variations in sex ratios at birth have long been and remain an important area of inquiry for evolutionary biologists. Several hypotheses have been advanced to specifically address the role of psychological distress as a cause of fetal sex, including proposals that parental hormone levels at the time of conception select for sex (James, 2010) or that exposure to stressors during gestation increases the risk of non-random fetal loss (Catalano et al., 2009). Together with evidence of a female bias in offspring sex among women occupying 'high stress' jobs (Ruckstuhl et al., 2010), these proposals suggest that the sex differences in maternal stress physiology may be the cause of and not the result of fetal sex. Nevertheless, given that we did not observe reliable differences in psychological distress between women carrying male versus female fetuses, we think it unlikely that these 'reverse' causality proposals can account for our results.

We note that our sample represents a healthy and relatively high SES population, and although there was significant variability in self-reported psychological distress, the overall level of psychological distress was relatively low, as would be expected in a community sample. Although it is tempting to assume that these findings would be exaggerated among women with greater levels of distress, this remains an important consideration for future research.

Finally, genetic factors that influence fetal growth cannot be ruled out in the present analysis. However, it is known that fetal growth is relatively more sensitive to the fetal environment compared to genetic contribution, and this environment is primarily determined by maternal physiology and placental function (Gluckman and Hanson, 2004).

The data we present offer important new insight into sex difference in the regulation of maternal stress physiology during pregnancy and their associations with birth weight. Future studies examining sex differences in the effects of exposure to prenatal psychological distress on fetal and child development should address the possibility that sex difference may derive from both the developmental program of the fetus and from maternal patterns of ANS and HPA axis function. Although the fetus and the maternal milieu are clearly separable sources of developmental programming, fetal-maternal communication is complex. In the context of the broader developmental programming literature, our findings suggest reciprocal regulation of maternal and fetal programming. Fetal sex contributes to regulation of maternal stress physiology, which in turn feeds back as a relevant regulatory signal for fetal development. Bi-directional communication between the mother and fetus suggest that the concept of fetal programming should be expanded to include the effects of the fetus on its own programming milieu.

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Table 1. Means (SD) or percentages for study variables and sample characteristics.

T1 (M = 14.9 weeks gestation; SD = 3.7; n = 291)								
Waking (n = 517)		Waking+30 (n = 532)		Mid AM (n = 560)		PM (n = 575)		
<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	
Cortisol (µg/dl)	.45(.18)	.45(.20)	.57(.24)	.53(.23)	.20(.09)	.22(.16)	.09(.05)	.10(.06)
sAA (U/ml)	85.10(78.6)	85.21(83.4)	54.28(46.94)	51.41(47.49)	94.98(77.15)	96.45(77.77)	88.95(70.82)	96.02(80.11)
T2 (M = 32.4 weeks gestation; SD = 1.0; n = 259)								
Waking (n = 462)		Waking+30 (n = 481)		Mid AM (n = 494)		PM (n = 501)		
<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	<u>Male</u>	<u>Female</u>	
Cortisol (µg/dl)	.52(.21)	.50(.21)	.72(.30)	.65(.27)	.33(.12)	.34(.16)	.16(.09)	.16(.11)
sAA (U/ml)	98.22(79.7)	94.29(85.8)	66.52(55.74)	57.67(47.32)	106.58(84.4)	108.78(85.2)	103.89(80.9)	101.23(83.3)
	<u>Male</u>	<u>Female</u>						
Psychological Distress (composite)								
EDS (depression)								
PSAS (pregnancy anxiety)								
SCL-90R (anxiety)								
Maternal age (years)								
Pre-pregnancy BMI (kg/m ²)								
Nulliparous								

Obstetric Risk Conditions	15.2%	17.9%
Income		
< \$40,000	11.0%	6.7%
\$40,000 -99,000	34.2%	38.1%
> \$100,000	54.8%	55.2%
Education		
High school or less	10.9%	10.5%
Trade/technical	17.0%	21.8%
University	72.1%	67.7%

Note: EDS = Edinburgh Depression Scale, PSAS = Pregnancy Specific Anxiety Scale, SCL-90R = Symptom Checklist 90 Revised

Table S1. Effects of fetal sex on the maternal sAA and cortisol awakening responses.

Fixed Effects	sAA Awakening Response			Cortisol Awakening Response		
	Estimate (SE)	t-ratio	p	Estimate (SE)	t-ratio	p
Intercept	4.146 (.090)	46.05	<.001	-.820 (.035)	23.51	<.001
Parity	.011 (.061)	.18	.854	-.019 (.025)	.78	.439
Pre-pregnancy BMI	.003 (.008)	.32	.750	-.005 (.004)	1.33	.186
Maternal age	.012 (.013)	.93	.356	.002 (.005)	.36	.718
Obstetric risk conditions	.147 (.141)	1.04	.298	.039 (.047)	.82	.411
Psychological distress	-.056 (.060)	.93	.356	-.067 (.021)	3.22	.001
Fetal Sex	-.085 (.110)	.78	.437	-.032 (.043)	.76	.450
Gestational week	.007 (.004)	1.62	.105	.008 (.002)	3.419	.001
Gestational week*fetal sex	-.0004 (.006)	.072	.943	-.002 (.003)	.53	.594
Linear slope (centered at Males)	-.813 (.091)	8.93	<.001	.499 (.043)	11.75	<.001
Change in linear slope for Females	.021 (.125)	.17	.863	-.058 (.067)	.857	.392
Gestational week	.009 (.008)	1.23	.222	.008 (.004)	2.33	.021
Gestational week*fetal sex	-.005 (.011)	.48	.631	.002 (.005)	.30	.764

Table S2. Effects of fetal sex on maternal daytime sAA and cortisol growth trajectories.

Fixed Effects	sAA			Cortisol		
	Estimate (SE)	t-ratio	p	Estimate (SE)	t-ratio	p
Intercept	3.656 (.094)	39.97	<.001	-.487 (.029)	16.61	<.001

Parity	.051 (.061)	.84	.403	-.058 (.018)	3.05	.003
Pre-pregnancy BMI	.006 (.008)	.71	.479	-.005 (.002)	2.26	.025
Maternal age	.010 (.012)	.84	.401	.006 (.004)	1.69	.091
Obstetric Risk Conditions	.057 (.132)	.43	.666	.042 (.032)	1.32	.189
Psychological distress	-.048 (.059)	.82	.414	-.028 (.015)	1.92	.056
Fetal Sex	-.100 (.108)	.92	.361	-.067 (.037)	1.81	.072
Gestational week	.011 (.003)	3.62	<.001	.015 (.002)	7.21	<.001
Gestational week*fetal sex	.0004 (.004)	.088	.930	-.003 (.003)	.94	.348
Linear slope (centered at Males)	.162 (.012)	13.34	<.001	-.253 (.009)	28.98	<.001
Change in linear slope for Females	.040 (.018)	2.21	.028	.039 (.013)	2.95	.003
Gestational week	-.0008 (.001)	.78	.434	.005 (.0007)	6.84	<.001
Gestational week *fetal sex	.00005 (.002)	.03	.98	-.002 (.001)	1.56	.119
Quadratic slope (centered at Males)	-.009 (.001)	12.02	<.001	.009 (.0006)	16.58	<.001
Change in quadratic slope for Females	-.002 (.001)	2.18	.030	-.002 (.0008)	2.79	.006
Gestational week	.00006 (.00007)	.83	.409	-.0003 (.00005)	5.37	<.001
Gestational week *fetal sex	-.00005 (.0001)	.47	.640	.0001 (.00007)	1.61	.108

Table S3. Associations between the maternal sAA and cortisol awakening responses and infant birth weight percentile.

Fixed Effects	sAA Awakening Response			Cortisol Awakening Response		
	Estimate (SE)	t-ratio	p	Estimate (SE)	t-ratio	p
Intercept	4.108 (.079)	51.99	<.001	-.839 (.029)	29.17	<.001
Parity	.006 (.061)	.10	.921	-.014 (.024)	.58	.565
Pre-pregnancy BMI	.001 (.008)	.17	.864	-.005 (.003)	1.46	.147
Maternal age	.014 (.013)	1.07	.287	.003 (.005)	.52	.603
Obstetric Risk Conditions	.176 (.140)	1.25	.210	.042 (.045)	.91	.362
Psychological distress	-.051 (.060)	.85	.398	-.065 (.022)	3.03	.003
Gestational week	.009 (.002)	4.50	<.001	.011 (.001)	8.71	<.001
Birth weight percentile	.003 (.002)	2.02	.044	.0005 (.0008)	.62	.536
Linear slope	-.807 (.063)	12.73	<.001	.464 (.033)	13.95	<.001
Effect of birth weight on linear slope	-.004 (.002)	2.01	.045	-.001 (.001)	.84	.402

Table S4. Associations between maternal daytime cortisol and sAA trajectories and infant birth weight percentile.

Fixed Effects	Daytime sAA			Daytime Cortisol		
	Estimate (SE)	t-ratio	p	Estimate (SE)	t-ratio	p
Intercept	4.331 (.083)	52.27	<.001	-1.649 (.024)	68.79	<.001
Parity	.051 (.061)	.84	.403	-.057 (.020)	2.85	.005
Pre-pregnancy BMI	.006 (.008)	.72	.471	-.005 (.002)	2.19	.029
Maternal age	.011 (.012)	.92	.357	.006 (.004)	1.62	.107
Obstetric Risk Conditions	.061 (.135)	.45	.650	.034 (.031)	1.09	.277
Psychological distress	-.047 (.059)	.80	.426	-.030 (.015)	2.01	.045
Gestational week	.009 (.002)	5.41	<.001	.024 (.001)	24.17	<.001
Birth weight percentile	.0007 (.002)	.41	.681	-.001 (.001)	.89	.374
Linear slope	.058 (.003)	18.42	<.001	-.135 (.002)	62.64	<.001
Effect of birth weight on linear slope	-.00003 (.0001)	.26	.796	-.0002 (.00007)	2.25	.028
Quadratic slope	-.010 (.001)	16.56	<.001	.008 (.0004)	19.02	<.001
Effect of birth weight on quadratic slope	-.00001 (.00002)	.31	.754	.000001 (.00002)	.04	.968

Figure 1. Daytime sAA slopes in pregnant women as a function of fetal sex.

Figure 2. Daytime cortisol trajectories in pregnant women as a function of fetal sex.

Figure 3. Daytime cortisol trajectories in pregnant women as a function of birth weight percentile.

Note: Low and high birth weight are graphed at the 5th percentile.