RUNNING HEAD: INFANT CORTISOL REACTIVITY

Sexually Dimorphic and Interactive Effects of Prenatal Maternal Cortisol and Psychological Distress on Infant Cortisol Reactivity

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Abstract

In utero exposure to maternal psychological distress is a risk factor for developmental psychopathology and these effects are believed to occur, in part, via dysregulation of the maternal and fetal hypothalamic-adrenal-pituitary (HPA) axes. Nevertheless, only a few human studies have directly assessed the effects of prenatal cortisol exposure on infant cortisol reactivity and none have investigated sex differences or potential interactions between prenatal cortisol and psychological distress. Here we report on a prospective longitudinal investigation (N=236) of in utero exposure to maternal cortisol and distress in a relatively high SES and low risk population to determine whether these exposures interact in their effects on infant (*M* age=3.0 months; Range=2.3-5.0; 51.9% male) cortisol reactivity and whether there are sex differences in these effects. Results revealed both sexually dimorphic and interactive effects of prenatal cortisol and distress, even after controlling for postnatal distress. In general, blunted reactivity in females was associated with exposure to high maternal distress and flattened patterns of diurnal maternal cortisol whereas blunted reactivity in males was associated with exposure to steeper morning increases and daytime decreases in maternal cortisol. The findings suggest that sex differences in the effects of prenatal cortisol and distress on infant cortisol reactivity are a plausible mechanism by which maternal experiences during pregnancy contribute to sex differences in the development of psychopathology.

Introduction

Fetal exposure to endogenous maternal glucocorticoids during gestation has broad and enduring effects on offspring development (Mina & Reynolds, 2014). In animal models of prenatal stress, glucocorticoid exposure (both endogenous and exogenous) influences the development of the fetal hypothalamic-adrenal-pituitary (HPA) axis function and this in turn leads to adaptation at every level of development from cells to behaviour (Cottrell & Seckl, 2009; Harris & Seckl, 2011). In utero exposure to maternal glucocorticoids is believed to affect fetal development through structural and functional reorganization of physiological systems, such as the HPA axis, in a process that has been described as fetal or developmental programming (Barker, 2004; Burton & Fowden, 2012; Sandovici, Hoelle, Angiolini, & Constância, 2012). Such programming operates as a form of non-genomic micro evolution that allows for rapid adaptation to changing environmental conditions.

According to the developmental origins hypothesis, glucocorticoids transmitted from mother to fetus during gestation convey useful information about the state of the external world and this information is exploited by the fetus to optimize development in preparation for survival and success in the postnatal environment (Bateson et al., 2004; Ellis & Del Giudice, 2013; Gluckman et al., 2009). Both animal and human studies suggests that male and female fetuses incorporate these signals in unique ways that result in sexually dimorphic outcomes (Sandman, Glynn, & Davis, 2013). For example, maternal distress and elevated cortisol during pregnancy may result in accelerated development in males but more variable (sometimes accelerated and sometimes reduced) in females (Doyle et al., 2015). Furthermore, several recent studies have shown that maternal cortisol production during pregnancy is itself influenced by fetal sex, with higher levels associated with female sex, especially toward the end of pregnancy (DiPietro,

Costigan, Kivlighan, Chen, & Laudenslager, 2011; Giesbrecht, Campbell, & Letourneau, 2015). Nevertheless, few human studies have evaluated the links between in utero cortisol exposure and infant HPA axis and none have evaluated whether this association depends on levels of psychological distress or fetal sex. In the current study we address these gaps.

During pregnancy, cortisol passes through the placenta in sufficient quantity to alter fetal development, despite partial blockade by the enzyme 11 beta hydroxysteriod dehydrogenase type 2 (Gitau, Cameron, Fisk, & Glover, 1998; Seckl, 2008). Psychological distress is a potent stimulant for the HPA axis in both non-pregnant and pregnant humans (de Weerth, Wied, Jansen, & Buitelaar, 2007; Giesbrecht, Campbell, Letourneau, & Kaplan, 2013; Giesbrecht, Campbell, Letourneau, Kooistra, & Kaplan, 2012; Nierop et al., 2006) and cortisol is widely cited as a plausible biological mechanism whereby maternal experiences of psychological distress are transmitted to the fetus (Mina & Reynolds, 2014). For these reasons, cortisol is proposed as a central mechanism for fetal programming and indeed animal models have convincingly shown that increases in maternal stress during pregnancy leads to behavioural and cognitive changes that influence the overall developmental potential of the offspring (Bale et al., 2010; Mina & Reynolds, 2014). It is important to note that the relationship between prenatal stress and offspring outcomes may depend on both the strength and timing of the prenatal exposure. There is some evidence that mild elevations in stress and cortisol, at least within otherwise healthy populations, may be associated with advanced fetal and child maturation (DiPietro, Novak, Costigan, Atella, & Reusing, 2006; Doyle et al., 2015). Negative effects of prenatal stress and cortisol exposures are most consistently observed when exposures occur early in development and when the levels of exposure are high (Davis & Sandman, 2010; Laplante et al., 2004), and potentially also at very low levels of exposure (Dipietro, 2012). Many of the effects on offspring

development are believed to result from or be exacerbated by hyper-reactivity or hypo-reactivity of the infant HPA axis following in utero exposure to maternal stress.

Prenatal maternal distress and infant HPA axis outcomes

Animal studies have shown that prenatal stress has both species-specific and sex-specific effects (Bale, 2011). For example, among guinea pigs chronic prenatal stress leads to blunted or hypo-reactivity in the HPA-axis response to stress exposure (Emack, Kostaki, Walker, & Matthews, 2008) whereas among rhesus monkeys (Coe et al., 2003) and rats prenatal stress leads to HPA axis hyper-reactivity, although in the case of rats these effects were found only for female offspring (Emack et al., 2008; Szuran, Pliska, Pokorny, & Welzl, 2000; Weinstock, 2001; Welberg & Seckl, 2001). A common theme across species and sex is persistent hyperactivity of *basal* HPA axis function, although the size of this effect is somewhat dependent on the timing and severity of the prenatal exposure (Welberg & Seckl, 2001). Human studies have also shown elevations in basal cortisol along with flattening of the daytime cortisol trajectory following in utero exposure to maternal psychological distress (Brennan et al., 2008; Laurent et al., 2013; O'Connor et al., 2005; O'Donnell et al., 2012; Vedhara et al., 2012; Yehuda et al., 2005), although several studies have reported lower basal levels among infants whose mothers had a history of abuse or depression as a child (Azar, Paquette, Zoccolillo, Baltzer, & Tremblay, 2007; Brand et al., 2010). The later findings highlight the special significance of early life adversity not only for individuals but also for their offspring. Chronic elevation and flat daytime cortisol patterns are risk markers for psychopathology (Gunnar & Vazquez, 2001; Tarullo & Gunnar, 2006).

Studies assessing the associations between in utero exposure to maternal psychological distress and children's cortisol responses to stressors have not produced a consistent set of

results. This is best exemplified in a study by Tollenaar and colleagues (Tollenaar, Beijers, Jansen, Riksen-Walraven, & de Weerth, 2011) who reported that prenatal maternal distress predicted infant cortisol reactivity but the direction of the effect varied by child age. Pregnancy-specific anxiety, but not general measures of anxiety or stress, was associated with *heightened* cortisol response to a bathing routine at 5 weeks of age but at 8 weeks and 12 months it was associated with *reduced* cortisol responses to a vaccination and maternal separation, respectively. Similarly, other studies have reported that prenatal depression and/or anxiety were associated (Azar et al., 2007; Brand et al., 2010; Vedhara et al., 2012; Yong Ping et al., 2015) or were not associated (Davis, Glynn, Waffarn, & Sandman, 2011) with increased offspring cortisol reactivity. Of these studies, only two reported testing for sex by psychological distress exposure interactions with both studies suggesting greater reactivity in females compared to males (Vedhara et al., 2012; Yong Ping et al., 2015). Nevertheless, previous studies have not examined sexually dimorphic effects of prenatal distress in the context of concurrent cortisol exposure and this is a significant limitation because it is known that prenatal psychological distress and cortisol have additive effects on offspring outcomes (Davis & Sandman, 2010).

Prenatal maternal HPA axis and infant HPA axis outcomes

Whereas many studies have examined the links between prenatal distress and children's cortisol, relatively few human studies have assessed the associations between prenatal cortisol exposure and children's HPA axis function. Of the six studies that we located, three reported positive associations between prenatal cortisol exposure and children's HPA axis function (Davis et al., 2011; Gutteling, de Weerth, & Buitelaar, 2005; Gutteling, de Weerth, & Buitelaar, 2004), one reported a negative association (O'Connor, Bergman, Sarkar, & Glover, 2013), and two studies found no association (de Weerth, Buitelaar, & Beijers, 2013; Tollenaar et al., 2011). One

additional study found that maternal hair cortisol during pregnancy was positively associated with offspring hair cortisol at age 1, and 3 but not 5 and 8, although this finding is more directly relevant to basal HPA axis function than to HPA axis reactivity (Karlen, Frostell, Theodorsson, Faresjo, & Ludvigsson, 2013).

These studies were all of relatively high quality and included appropriate controls for potential confounding variables in statistical analyses. Nevertheless, differences with regard to timing of the maternal cortisol measure (both within the day and within pregnancy), differences in the type of prenatal cortisol exposure (e.g., area under the curve, CAR, amniotic fluid) and differences in the type of infant stressor may have contributed to the disparate findings. Furthermore, two key considerations have been neglected in these studies. First, child sex has not been included as a potential moderator. This is a significant limitation because sex is known to moderate the effects of prenatal distress on a variety of outcomes in children (Clifton, 2010; Davis & Pfaff, 2014; Sandman et al., 2013), including HPA axis function (Vedhara et al., 2012; Yong Ping et al., 2015), and because maternal cortisol secretion during pregnancy differs as a function of fetal sex (Giesbrecht et al., 2015). Second, all previous studies have considered prenatal distress and cortisol as independent predictors. Although this approach has yielded valuable insight regarding the unique effects of psychological and biological predictors on infant outcomes it does not address the possibility that prenatal distress and cortisol have interactive (and possibly synergistic) effects on children's HPA axis. Because in utero cortisol exposure is a plausible biological mechanism by which maternal experiences of psychological distress become embedded in children's development and because HPA-axis reactivity mediates the effects of the environment on children's development, studies assessing sex differences in the effects of prenatal cortisol and psychological distress exposures on infant cortisol reactivity have the

potential to advance our understanding of the developmental origins of sex differences in developmental psychopathology.

Current Study

The current prospective study, in a relatively high SES and low risk sample, set out to determine whether the associations between prenatal maternal cortisol and infant cortisol reactivity are moderated by infant sex and prenatal distress. Given evidence that the timing of exposure may affect infant outcomes (Tollenaar et al., 2011; Vedhara et al., 2012), we assessed pregnant women as early in pregnancy as possible, but in all cases prior to 22 weeks gestation and again near the end of pregnancy. Infant HPA axis function was assessed at 3-months postpartum using a blood draw as the stressor. Building upon the strength of the previous studies, we included appropriate covariates to rule out effects of the postnatal environment. Our aims were to replicate previous findings of sex differences in the association between psychological distress and infant cortisol reactivity, to determine whether the association between prenatal maternal cortisol and infant cortisol reactivity is moderated by infant sex, to determine whether the combined effects of prenatal cortisol and distress are moderated by infant sex, and to determine whether these associations are consistent across different points of gestation.

Methods

Sample

Participants were 294 women enrolled in an ongoing prospective cohort study, the Fetal Programming study, which is a community sample of volunteers recruited in 2011 and 2012. Women were included if they had a singleton pregnancy, were less than 22 weeks of gestation at the first study visit, and were 18 years of age or older. Women were excluded if they smoked or consumed alcohol during pregnancy, were being treated with a synthetic glucocorticoid, or had

known fetal complications at time of study entry. Because gestational age at birth and birthweight are associated with infant HPA axis function (Buss et al., 2012; Glover, Miles, Matta, Modi, & Stevenson, 2005), we made an a priori decision to exclude from current analysis infants with low birthweight (< 2500 g) or preterm birth (< 37 weeks gestation). The sample was a relatively well-educated (85.7 % had education beyond high school), financially stable (52.7% had household income ≥ $100, 000 CAD/annum, 8.5% had household income < $39.999 CAD/annum), married or common-law (94.9 %), mature (mean age = 31.4 years, SD=3.8) and primiparious (51.7%). The majority were non-Hispanic white (77.2%); the remainder were Asian (9.5%), Latin American (3.7%), Arab (2.0%), Japanese (1.4%), Filipino (1.0%), or other (5.2%). The sex ratio of infants favored boys (*n* = 118; 51.9%), consistent with the worldwide birth ratio of 51.7% males and 48.3% females (Hesketh & Xing, 2006).

Procedures and Measures

Saliva collection during pregnancy. Participants collected a series of diurnal saliva samples at two time points in pregnancy: T1 = two days in early pregnancy (mean = 14.9 weeks GA, range = 5.9 – 21.9 weeks GA), and T2 = two days in late pregnancy (mean = 32.5 weeks GA, range = 27.1 – 36.7 weeks GA). At each time point, women self-collected saliva at home during weekdays on the following schedule: upon waking, 30 minutes after waking, at 1130h, and at 2100h. On each sampling day, participants collected the waking sample immediately upon waking and then initiated a 30 minute timer on a personal digital assistant (PDA). This procedure allowed for precise timing of the 30 min post-waking 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 first provided a code corresponding to a pre-labeled saliva tube and instructed the participant to place the saliva roll under her tongue.

Participants then completed the PDA-administered psychological distress questionnaire during saliva collection. Time of each assessment was recorded by the PDA, permitting precise modeling of diurnal patterns. To facilitate adherence to the study protocol, the PDA was programmed to allow a 20 minute response window following the signal, after which the questionnaire was no longer available.

Whole saliva was obtained from under the tongue using the SalivaBio Oral Swab (Carlsbad, CA). Mothers were asked to refrain from consuming food, caffeine, citric drinks and dairy, and to avoid vigorous exercise (e.g., running or playing sports) or brushing their teeth within 30 minutes prior to saliva collection and to report adherence to these guidelines. Saliva samples were stored at -20°C in participant home freezers until they were returned to the lab where they were they remained frozen at -80°C until they were shipped frozen to Salimetrics, State College, PA.

Infant saliva collection. Infant cortisol was assessed at age 3 months before (baseline) and after (5 min, 20 min, and 40 min) a blood draw. Whole saliva was collected using sorbettes (BD Visispear™) which have been validated for cortisol collection (de Weerth, Jansen, Vos, Maitimu, & Lentjes, 2007).

Cortisol Assay. All infant and maternal samples were assayed for salivary cortisol using the Salimetrics enzyme immunoassay. It has a lower limit of sensitivity of 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 serial dilution are 100.8% and 91.7%. A random 10% of samples were assayed in duplicate to confirm reliability; the intra-assay coefficient of variation and correlation coefficient between the duplicate tests for the

maternal samples were *cv* = .042 and *r* = .99, respectively and for the infant samples *cv* = .035 and *r* = .99, respectively. Mean values from duplicate samples were used for analysis.

Prenatal Maternal Psychological Distress. Maternal distress during pregnancy was assessed simultaneous to saliva sample collection using 9 indicators of psychological distress (e.g., I felt tense, I felt discouraged, I had many worries) taken from the Profile of Mood States (POMS) (McNair & Heuchert, 2007) and adapted for administration on the PDA following procedures previously described (Cranford et al., 2006; Giesbrecht et al., 2012). Participants rated each item on a 5-point Likert scale from not at all to extremely, based on their feelings during the previous 30-minutes. The mean of the 9 items was used to calculate a psychological distress score for each person (possible range 0-4) with higher scores indicting more distress. Reliability and validity of a short PDA-administered version of the POMS is supported by previous research with participants experiencing a major life stressor (Cranford et al., 2006) and with pregnant women (Giesbrecht et al., 2012). In the current study the 9-item distress measure had a Cronbach's alpha of .87.

Infant stressor. The infant blood draw at 3 months was completed by a certified pediatric phlebotomist using either venipuncture or heel stick. Parents were allowed to choose the method to increase their willingness to complete this portion of the study. For venipuncture, a #2 butterfly (winged infusion 12") needle or 25G x ¾" standard needle was used in the forearm to collect 3-4 mL of blood. Heel stick was performed with a 1.0mm (2.5WX1D mm) lance and enough blood was squeezed from the heel to blot a 1x1cm strip of chromatography filter paper. Blood samples obtained using these procedures were not analyzed for the current report.

Data Reduction

Maternal Psychological Distress. For both the early and late pregnancy time points (T1 and T2, respectively), a mean psychological distress score was calculated by aggregating all distress items from all sampling moments. The Cronbach's alpha for the distress measure was .88 at T1 and .85 at T2.

Maternal Cortisol. Three summary measures of maternal pregnancy cortisol were derived from the diurnal saliva samples to capture different aspects of HPA axis function. Samples collected at waking and 30 min post waking were used to calculate the cortisol awakening response (CAR) using the trapezoid method for area under the curve increase (Pruessner, Kirschbaum, Meinlschmid, & Hellhammer, 2003) as a measure of the morning increase in cortisol. Samples were excluded from this analysis if they were taken more than 15 minutes after waking (for the waking sample) or more than 50 minutes after waking for the second sample (Okun et al., 2010; Wust et al., 2000). A measure of total cortisol secretion was obtained from samples collected at waking, 1130h, and 2100h and calculated using the trapezoid method for the area under the curve from ground (AUCg) (Pruessner et al., 2003). Finally, the daytime slope was calculated by fitting a regression slope through the cortisol data for each individual to describe the daytime cortisol decline (Fekedulegn et al., 2007). The daytime slope is expected to be negative (and in fact it was in all cases in our sample) thus values closer to zero indicate a flatter slope whereas more negative numbers indicate a stronger decline. The daytime slope included the 30 min post waking, 1130h and the 2100h samples.

Statistical Procedures

Data Screening. Of the 294 women enrolled during pregnancy, 255 returned for the 3-month infant assessment. Of these, 19 mothers or infants were excluded from analysis because of failure to follow the study protocol (n=1), and either low birthweight (< 2500 g) or preterm birth

(< 37 weeks gestation) (n = 18). Thus, the final sample for analysis was 236. Women in the final sample did not differ from the original sample on any demographic or study variables, with the exception of daytime slope at T2; women not in the final sample had a flatter daytime slope compared to women in the final sample, t (292) = 2.36, p = .019. The data was screened for potential outliers and values that exceeded a z-score of 3.29 were adjusted according to recommendations (Tabachnick & Fidell, 2012). No more than four values (1.5%) were adjusted for any variable.

Analytic Models. Multilevel modeling was used to model trajectories of infant cortisol as a function of prenatal maternal cortisol, distress, and sex. Multilevel models have advantages over other repeated measures approaches in their ability to accommodate individual timing of samples, they flexibly accommodate missing data, and they allow for control of initial values effects (Kristjansson, Kircher, & Webb, 2007; Llabre, Spitzer, Siegel, Saab, & Schneiderman, 2004). A two-level model with random intercept and slopes was fit to the infant cortisol data using the Mixed procedure in SPSS 22.0. The outcome was the log of infant cortisol. At level 1, both the linear and quadratic effects of time were included to allow curvilinear trajectories in infant cortisol (cortisol increases are expected to slow or reverse between 20 and 40 minutes posts stressor) and to allow for individual differences in peak cortisol (maximum levels are delayed in some infants). Time was centered at baseline, thus the intercept in the mixed models refers to cortisol levels at baseline. The primary variables of interest were maternal prenatal cortisol, prenatal distress and infant sex, which were included in the level 2 submodel for the intercept and linear slope. Maternal prenatal cortisol and distress were centered at the sample means and sex was centered at males. Selection of covariates, random effects and error structure

was based upon model fit as assessed by -2 log likelihood. Separate analyses were conducted for the T1 and T2 exposures to determine if exposure timing influenced the results.

Covariates. We included in our models covariates identified in previous studies (Buss et al., 2012; Glover et al., 2005; Granger, Hibel, Fortunato, & Kapelewski, 2009) as potential confounders of the prenatal cortisol – infant cortisol association. In preliminary models, infant age at assessment, infant birthweight, blood draw method (venipuncture vs. heel stick), infant sleeping versus awake on the car ride to the appointment, maternal or infant medication use, and maternal pre-pregnancy BMI were not associated with infant cortisol (all *p*s > .15) and were therefore excluded from further analysis. All models presented here included the following covariates: gestational week of pregnancy for maternal cortisol collection, arrival time at the laboratory, infant age at birth, infant negative affect, and maternal postnatal psychological distress (depression and anxiety), which was included to control for current level of distress. Infant negative affect in the period from arrival at the laboratory until the blood draw was rated by a trained research assistant using the negative affect scale of the Behavior Rating Scales of the Bayley Scales of Infant Development 2nd edition (Bayley, 1993), which assesses the amount and intensity of infant fussing/crying on a 5-point scale from "no negative affect displayed" to "three or more intense, heightened, or prolonged displays of negative affect". Postnatal depression was assessed via the10-item, self-report *Edinburgh Postnatal Depression Scale* (EPDS) (Cox, Holden, & Sagovsky, 1987). Scores range from 0-30, with scores above 12 strongly correlated with physician diagnosis of Major Depressive Disorder (Jomeen & Martin, 2007). In our study, the EPDS Cronbach alpha was adequate, $\alpha = .78$. Postnatal anxiety was assessed using the *Symptom Checklist-90*-R (SCL-90-R) (Derogatis, 1994), which is a multidimensional symptom inventory designed to reflect psychological symptom patterns. Only the 10-item anxiety scale

was used in the current report; this anxiety scale has good convergent and divergent validity (Morgan et al., 1998) and adequately discriminates between clinical and non-clinical samples (Bonicatto et al., 1997; Holi et al., 1998). In our study, the SCL-90-R anxiety scale Cronbach alpha was adequate, $\alpha = .79$. The postnatal depression and anxiety measures were correlated $r = .50$ and therefore were z-transformed and combined to reduce the number of covariates included in the model. All covariates were centered at the sample mean, with the exception of infant negative affect which was centered at 'no negative affect'.

Results

Descriptive Results

Descriptive statistics displayed in Table 1 reveal several expected results. First, infant cortisol levels at baseline and 5 min post blood draw were not different,[1] $t(70) = 1.19$, $p = .237$, but there was a clear increase in cortisol 20 minutes post stressor, $t(247) = 6.53$, $p < .001$, and despite a small decline by 40 minutes post stressor infant cortisol remained significantly elevated over baseline, $t(243) = 2.28$, $p = .024$. The absolute cortisol increase from baseline to 20 minutes post stressor was .132 ug/dl. Second, maternal cortisol increased from T1 to T2, $t(268) = 21.09$, $p < .001$ for AUCg and $t(268) = 5.20$, $p < .001$ for CAR. There were no differences in distress or daytime slope between T1 and T2.

*Replication of Prenatal Maternal Distress*Sex Association*

Maternal distress at T1 was associated with sex differences in baseline cortisol, $\beta = .328$, $p = .033$, but not with increase in cortisol in response to the stressor, $\beta = -.162$, $p = .508$ (see Model 1, Table 2). As shown in Figure 1, females exposed to low stress and all males had similar baseline and slopes whereas females exposed to high distress had elevated baseline cortisol

[1] 5 min post blood draw was discontinued after the first 72 samples.

resulting in overall increased levels of cortisol as compared to males $F(1, 224) = 6.51$, $p = .011$. At T2, these sex differences were reduced to a marginal effect on baseline cortisol, $\beta = .116$, $p = .064$, and a non-significant effect for infant cortisol increases, $\beta = -.134$, $p = .165$ (see Model 1, Supplementary Table 1).

*Effects of Prenatal Maternal Cortisol*Sex*

CAR. The associations between the maternal CAR and infant cortisol reactivity at both T1 and T2 were moderated by infant sex (Model 2, Table 2). Note that because the results for T1 and T2 were similar, we have provided in Table 2 only the results for T1. Complete model results for T2 are available in supplementary Table 1. At T1, the interaction between CAR and sex was significant both for baseline infant cortisol, $\beta = -.033$, $p = .002$, and for the cortisol increase in response to the stressor, $\beta = .056$, $p = .005$. As shown in Figure 2, the effects of the maternal CAR were essentially opposite for males and females. For example, males exposed to a low CAR and females exposed to a high CAR both displayed large cortisol increases. Similar findings were observed for CAR T2, with significant CAR by sex interactions both at baseline, $\beta = -.027$, $p = .007$, and the increase over time, $\beta = .062$, $p < .001$ (see Model 2, Supplementary Table 1).

To probe the sex*CAR T1 effects, we calculated regions of significance using the web utility at www.quantpsy.org and described by Preacher and colleagues (Preacher, Curran, & Bauer, 2006). Male and female reactivity differed ($p < .05$) when maternal CAR T1 was below the 26^{th} percentile or above the 89^{th} percentile.

AUCg. There were no effects of maternal AUCg T1 or T2 on infant baseline or change over time and no interactions between AUCg and infant sex.

Daytime slopes. The results for daytime slope were substantially the same as those for the CAR. Effects were observed for maternal daytime slope at both T1 (see Model 3, Table 2) and T2 (see Model 3 Supplementary Table 1). Females exposed to a flatter maternal daytime slope had elevated baseline, β = -.052, p = .005, and smaller cortisol increases, β =.059, p = .046, compared to males. Similar findings were observed for daytime slope T2, although the effect at baseline was marginally significant, β = -.033, p = .075, but the increase over time was significant, β = .063, p = .033.

*Effects of Prenatal Maternal Cortisol*Distress*Sex*

In the next set of models we included both the maternal cortisol and distress variables and their interaction to determine if parental cortisol and distress interact in their effects on infant cortisol. Our interest was primarily in the interaction terms between cortisol, distress, and sex.

CAR. There was a 3-way interaction between CAR T1, distress, and infant sex for infant cortisol reactivity, β = -.377, p = .033, but not for baseline cortisol, β = .056, p = .608 (Table 2, Model 4). For clarity of presentation, the findings for Model 4 are displayed in two figures - Figure 3 displays the interaction between sex and CAR T1 at low distress exposure and Figure 4 at high distress exposure. At low levels of prenatal distress (Figure 3), CAR T1 had opposite effects on male and female reactivity. That is, greater cortisol reactivity was associated with high CAR T1 exposure among females but with low CAR T1 exposure among males. Likewise, blunted cortisol reactivity was associated with low CAR T1 exposure in females but with high CAR T1 exposure among males. In contrast, at high levels of prenatal stress (Figure 4) CAR T1 exposure had little effect on reactivity among males whereas females exposed to high CAR T1 had blunted reactivity and females exposed to lower CAR T1 had elevated reactivity. Exposure

to high distress and low CAR T1 was associated with significantly higher cortisol levels in the post stressor period among females compared to males, $F(1, 194) = 7.02$, $p = .009$.

For T2, the 3-way interactions between CAR, distress, and sex was not significant for either baseline or reactivity (Model 4, Supplementary Table 1). However, similar to the effects observed at T1, the 2-way interactions between sex and cortisol were significant for both baseline $\beta = -.046$, $p = .030$, and reactivity, $\beta = .090$, $p = .009$ even after controlling for the effects of prenatal distress.

AUCg. There were no 3-way interactions between AUCg, distress and sex for either T1 or T2.

Daytime slopes. The 3-way interactions between maternal daytime slope, prenatal stress and infant sex were not significant at either T1 (Model 5, Table 2) or T2 (Model 5, Supplementary Table 1). Nevertheless, all significant 2-way interactions observed in the individual models for psychological distress (Model 1) and daytime cortisol (Model 3) remained significant in the combined model. Similar effects were observed for the T2 model (Model 5, Supplementary Table 1), however with the exception of the daytime slope by sex interaction, all other significant effects at T1 became trends at T2.

Discussion

In this prospective longitudinal study, we observed sexually dimorphic effects of prenatal cortisol and distress exposure on infant cortisol response to a pain stressor. The most striking feature of these findings is that prenatal cortisol and distress exposure had essentially opposite effects on male and female cortisol reactivity. That is, a smaller maternal CAR or flatter daytime slope were associated with blunted cortisol responses in females whereas a larger CAR or steeper daytime slope were associated with blunted cortisol responses in males. When considering the

combined effects of prenatal cortisol and distress exposure, males appeared to be less affected than females. Females exposed to a smaller CAR or flatter daytime slope combined with high distress exposure showed elevated cortisol responses, whereas females exposed to a large CAR or steeper daytime slope and high distress showed a blunted response. Males, in contrast, had similar cortisol increases regardless of the combination of cortisol and distress exposures. These findings suggest that prenatal cortisol and distress have interactive and sexually dimorphic effects on fetal HPA axis development in higher SES, lower risk populations.

Altered cortisol reactivity is associated with a variety of stress-related diseases (e.g., hypertension) and psychiatric disorders (e.g., depression) (Bosch et al., 2012). In utero exposure to maternal distress and cortisol are associated with lifelong alterations of neuroendocrine, behavioral and cognitive functions (Van den Bergh et al., 2008; Weaver, 2009) and elevated risk for developing psychiatric, cardiovascular and metabolic disorders in later life, all of which are moderated by sex (Schlotz and Phillips, 2009). For example, a recent prospective investigation of prenatal cortisol exposure reported increased ten-year risk of coronary heart disease in adult offspring at age 42 (Stinson et al., 2015). The risk, however, was only for women. In the context of other research suggesting that the HPA axis is a central mediator for many health and disease outcomes (Del Giudice, Ellis, & Shirtcliff, 2011), our findings highlight the potential for lifelong effects related to prenatal cortisol exposure.

These findings may also have relevance to our understanding of sex-differences observed in the incidence and presentation of stress-related disorders. Davis and colleagues (Davis & Pfaff, 2014; Sandman et al., 2013) have argued that sex-specific fetal growth strategies result in greater adaptive flexibility for females in the short-term, especially under conditions of maternal distress, but increase the long-term risk for developmental psychopathology, with anxiety and

depression as two examples of stress-related psychopathology for which there are clear sex differences in presentation and prevalence (Altemus, Sarvaiya, & Neill Epperson, 2014). That patterns of HPA axis function in offspring may indeed link prenatal distress exposure to later psychopathology, at least for females, is supported by research showing that prenatal distress exposure is associated with a flattened diurnal cortisol profile in female but not male offspring during adolescence (Van den Bergh, Van Calster, Pinna Puissant, & Van Huffel, 2008).

The findings underscore the importance of including sex in studies assessing the effects of prenatal exposures on offspring HPA axis development. Although previous studies have not examined sex differences in offspring cortisol responses as a function of prenatal *cortisol* exposure, sex differences related to psychological distress exposure have been described; males tend to display blunted responses whereas females display increased responses to stressors (Vedhara et al., 2012; Yong Ping et al., 2015). Our findings partially replicated these studies in that we observed elevated cortisol responses among females prenatally exposed to higher distress but blunting of cortisol responses was observed only in reference to prenatal cortisol and was observed for both males and females, albeit under different levels of exposure. Our findings are also consistent with sexually dimorphic effects of prenatal exposure to exogenous glucocorticoids. Alexander and colleagues (2012) found that exogenous glucocorticoids (administered prenatally because of threatened preterm birth) were associated with heightened cortisol responses observed among females.

Our findings offer insight into the inconsistent pattern of results that have been observed across previous studies assessing the association between in utero cortisol exposure and infant cortisol reactivity. Ignoring sex difference essentially takes the average of the effects for males and females, and because the effects we observed were, in some cases, opposite for males and

females (see for example Figures 2 and 3) the results in any specific study will depend on the composition of the sample, including sex ratio. Indeed, of the previous studies reporting a positive association between prenatal cortisol and infant cortisol reactivity, the samples favored females (64%) although we note that the Davis et al. study (2011) had slightly more males (52.5%) even though they reported a positive association. Taken together, these findings suggest that sex differences in the effects of maternal cortisol and distress may essentially 'wash out' the overall effects when sex effects are not explicitly modelled.

Our findings of sexually dimorphic effects of prenatal cortisol on infant cortisol reactivity extend previous findings by demonstrating both the independent effects of prenatal cortisol and distress exposures and their interactive effects on the developing HPA axis. Models 1-3 (Table 2) show the individual effects of distress and cortisol whereas Models 4 and 5 show the unique and interactive effects. Importantly, all of the individual effects of prenatal distress and cortisol remained when both exposures were included in the same model, although in the case of Model 4 (CAR) the unique effect of psychological distress was marginal. These findings confirm that distress and cortisol contribute in unique ways to development of the fetal HPA axis (Davis & Sandman, 2010). However, these findings also suggest that cortisol cannot fully account for the effects of maternal distress on infant cortisol reactivity. Indeed, while animal studies have shown that maternal cortisol during pregnancy mediates the association between maternal distress and infant outcomes, there is a substantial human literature that has not found support for the potential association between maternal cortisol and child outcomes (de Weerth & Buitelaar, 2005; Zijlmans, Riksen-Walraven, & de Weerth, 2015). Based upon the current findings, we argue that overlooking fetal sex as a potential moderator may help to explain the lack of empirical support for the effects of prenatal stress exposures on child outcomes. Future research

will help to more fully explicate the effects of maternal distress on infant development by including sex within statistical models and by incorporating biomarkers for other stress response systems, such as the sympathetic and parasympathetic nervous systems.

The interactive effects of prenatal cortisol and distress exposures were, not surprisingly, complex. At low levels of prenatal distress, the effects of cortisol exposure were the same as those observed in the models where only cortisol was included (i.e., Models 1 and 2), perhaps reflecting the fact that the sample as a whole had relatively low levels of psychological distress. In contrast, at high levels of prenatal distress exposure, variation in maternal cortisol had little effect on males whereas females exposed to a small CAR and flatter daytime slopes had both elevated baseline and robust increases whereas females exposed to a large CAR or steeper daytime slopes had essentially no response to the stressor (Figure 4).

Consistent with the regions of significance analysis reported by Young Ping and colleagues (2015), infant cortisol levels differed as a function of maternal cortisol exposure only at the low and high ends of the maternal cortisol distribution. These findings support the notion that both too little and too much cortisol during gestation have important implications for fetal development. It is important to note, however, that low and high cortisol exposures present differential risk for the males and females. As shown in Figures 1 and 3, and taking blunting of cortisol responses as an indicator of a potentially problematic adaptation, males appear to be vulnerable to high cortisol exposures whereas females appear to be vulnerable to low exposures.

Across the three measure of cortisol during pregnancy, those that measured diurnal changes (i.e., the CAR and diurnal slope) were associated with infant HPA axis function whereas AUCg, a measure of total cortisol, was unrelated to infant cortisol. In the context of the Developmental Origins hypothesis, we speculate that the total amount of prenatal cortisol

exposure is a less useful signal to the fetus compared to the pattern of changes occurring within the maternal HPA axis. Absolute levels of cortisol may provide less information about the postnatal environment than do the fluctuations in cortisol. Some support for this notion can be derived from the frequently reported observation that cross-sectional correlations between psychological distress and cortisol are often null or very low whereas robust cortisol increases are observed following exposure to a stressor. This difference seems to indicate that overall measures of cortisol carry little information about an individual's psychological experience but dynamic changes in cortisol strongly reflect experience. During pregnancy, cortisol is dynamically related to interactions with the environment such that maternal experiences of psychological distress are related to both momentary increases in cortisol (Giesbrecht et al., 2012) and to overall changes in diurnal patterns (Obel et al., 2005). In this way, dynamic measures of cortisol represent signals to the developing fetus about maternal experience within her environment and the environment into which the fetus will be born. These findings suggest that researchers interested the effects of prenatal stress exposures on offspring HPA axis function should plan to collect dynamic measures of maternal HPA axis function, as opposed to single samples of blood, saliva or hair that can only provide overall estimates of secretory output.

In general, the timing of exposure during gestation had little effect on the outcomes. Nevertheless, the findings for earlier pregnancy were consistently stronger compared to those observed for later pregnancy, and the interaction between cortisol, distress and sex was significant only for earlier pregnancy. This is consistent with a common theme among developmental studies that biological systems are most sensitive to external influences during periods of rapid development (Bornstein, 1989). From this perspective, infant HPA axis function should be most strongly associated with exposures during the period around mid-gestation when

the fetal HPA axis becomes functional (Gitau, Fisk, Teixeira, Cameron, & Glover, 2001). Indeed, such effects were observed in a large population-based cohort of 1.1 million births in which the risk for stress-related disorders among offspring, especially males, was highest when exposure to severe life events occurred in the second trimester (Khashan et al., 2011). Nevertheless, several studies have found that stress and cortisol exposures in later gestation have more pronounced effects on the developing HPA axis compared to earlier exposures (Davis et al., 2011; Vedhara et al., 2012; Yehuda et al., 2005). The reasons for these discrepancies are not clear, however we note that the cortisol and distress exposures in our study are continuous over the period of gestation and not discrete as might be observed for a significant life event. For example, the experience of a natural disaster and associated stress would be confined to the third trimester if it initially occurred in the third trimester. Thus, in the context of our research, we do not expect strong timing effects.

Because the postnatal environment also appears to shape infant behavioral and physiological responses to stress (Bergman, Sarkar, Glover, & O'Connor, 2010), it is important to note that the effects we observed were independent of postnatal exposure to maternal anxiety and depression. Furthermore, the effects we observed were not explained by sociodemographic factors such as household income, maternal education, age, or pre-pregnancy BMI, or by characteristics of the infant such as negative affect immediately prior to the stressor or gestational age at birth. These findings strengthen the hypothesis that prenatal exposures were independently associated with the sex differences we observed.

Strengths and Limitations

The strengths of this study include its prospective design, multiple prenatal assessments, the inclusion of both psychological measures of distress and cortisol, the inclusion of potential

confounders as covariates in our models, the consideration of previously overlooked sex differences, and adequate sample size to test for sex-specific effects. Nevertheless, the interpretation and implications of these findings are subject to several limitations. First, the participants are comprised of a community sample with relatively high family income and education. It should be noted that although the median household income in the sample was high, it is equivalent to the median household income of the greater metropolitan region in which the sample was recruited. Second, although a range of distress scores were observed in this sample, the sample as a whole reported overall levels of distress that were low. We note that the majority of previous studies assessing the links between maternal prenatal stress and infant outcomes have done so with community samples comparable in terms of socioeconomic status and distress to the current sample (see for example DiPietro, Kivlighan, Costigan, & Laudenslager, 2009; Jacobs et al., 2007). Third, it was not possible to schedule the blood draw at the same time of day across our sample or to use the same blood draw method for all infants. Nevertheless, neither time of day nor blood draw method was associated with infant cortisol in any of our models, and this decreases the possibility that it may have contributed to the results. Fourth, a longer period of acclimation to the lab and multiple baseline samples would have increased confidence that the baseline measures were an accurate reflection of true basal levels. Finally, it will be important in future studies to account for the effects of sex-specific placental responses to maternal distress. Placental sex differences in the expression of 11ßHSD (lower when the fetus is female; Mericq et al., 2009) and glucocorticoid receptors (higher in males; Saif et al., 2015) suggest that the placenta may regulate cortisol metabolism in sex specific ways, and thereby contribute to sex differences in development of the fetal HPA axis.

Conclusion

Results of the present study showed that the associations between maternal cortisol during pregnancy and infant cortisol reactivity are moderated by sex and by maternal psychological distress. These findings suggest that the effects of in utero cortisol exposure on HPA axis function in children is different for boys and girls and that these effects depend on simultaneous in utero exposure to maternal distress, at least for girls. Further investigation is needed to determine whether sex specific alteration of infant HPA axis function consequent to in utero cortisol and distress exposure is links these prenatal exposures to sex differences in the development of psychopathology.

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Table 1. *Descriptive Statistics Mean(SD) for Study Variables by Time point*

Variables	**Mean (Range or SD)**	**N**
Pre-pregnancy BMI	24.79 (15.69 – 44.79)	236
Infant GA at birth (weeks)	39.48 (37.0 – 42.0)	236
Infant birth weight (g)	3438.79 (2518 - 5210)	236
Laboratory arrival time	1215h (855h – 1911h)	236
Infant negative affect during period from arrival to blood draw	.47 (0 - 4)	236
Maternal Postnatal Depression	5.08 (0 - 19)	236
Maternal Postnatal Anxiety	2.21 (0 - 20)	236
Infant age (weeks)	12.26 (9.1 - 20)	236
Infant Cortisol (ug/dL)		
Baseline	.239 (.142)	231
5 min post stressor	.249 (.136)	72
20 min post stressor	.326 (.166)	232
40 min post stressor	.270 (.150)	228

	T1 (14.9 weeks GA)	**N**	**T2 (32.5 weeks GA)**	**N**
Maternal Prenatal Distress	1.36 (.98 – 2.61)	236	1.34 (.71 – 2.38)	236
Maternal Cortisol		236		236
CAR	.96 (1.72)	236	1.59 (1.79)	236
AUCg	155.02 (38.94)	236	215.04 (44.35)	236
Daytime Slope	-2.48 (1.02)	236	-2.54 (1.02)	236

Note: CAR = cortisol awakening response, AUCg = area under the curve from ground.

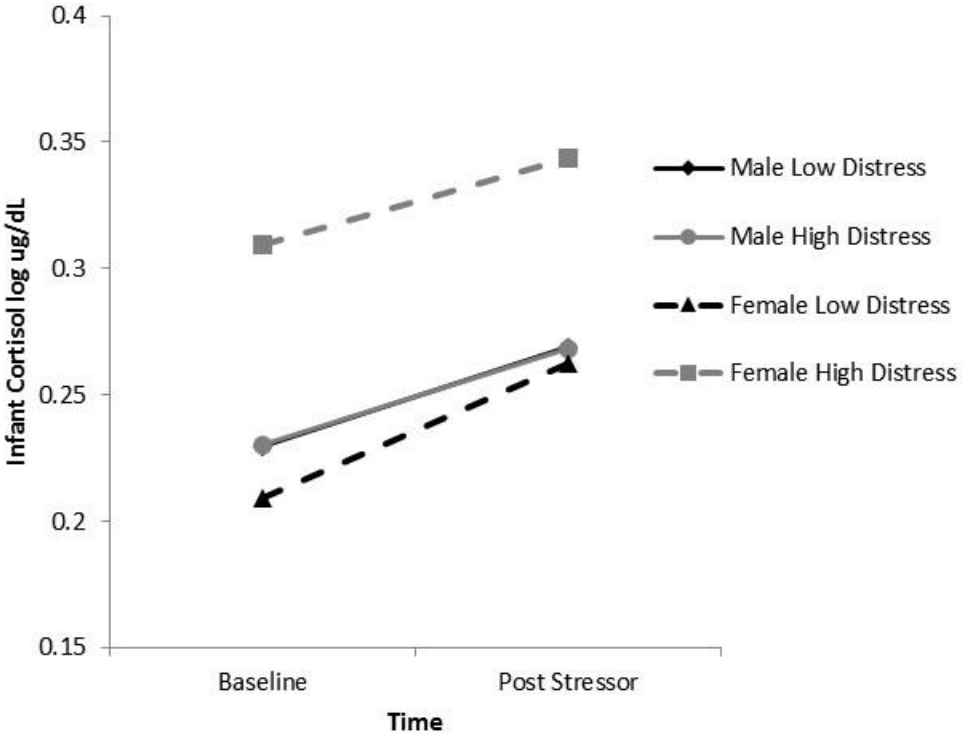
Table 2. HLM coefficients (SE) for prenatal cortisol T1, prenatal distress T1, and infant sex with infant cortisol reactivity as outcome.

	Model 1 (Psychological Distress)		Model 2 (CAR)		Model 3 (Daytime slope)		Model 4 (CAR *Distress)		Model 5 (Daytime slope*Distress)	
Fixed Effects	Estimate (SE)	*F*	Estimate (SE)	*F*	Estimate (SE)	*F*	Estimate (SE)	*F*	Estimate (SE)	*F*
Intercept	.230 (.014)	287.82***	.225 (.013)	286.35***	.228 (.013)	295.62***	.226 (.014)	273.25***	.228 (.013)	289.61***
Infant Sex	.021 (.018)	1.32	.013 (.017)	.54†	.013 (.018)	.56	.022 (.019)	1.34	.021 (.018)	1.30
Arrival Time	-.005 (.004)	2.05	-.006 (.004)	2.91†	-.005 (.004)	2.09	-.005 (.004)	1.76	-.005 (.004)	1.78
GA at birth	-.013 (.007)	3.97*	-.013 (.006)	3.95*	-.011 (.006)	3.04†	-.013 (.007)	3.91*	-.012 (.007)	3.26†
Negative Affect	.013 (.009)	2.00	.016 (.008)	3.76*	.016 (.009)	3.36†	.013 (.009)	2.25	.014 (.009)	2.41
Postnatal Distress	-.018 (.011)	2.95†	-.010 (.009)	1.35	-.010 (.009)	1.23	-.018 (.011)	2.80†	-.018 (.011)	2.91†
Maternal Cortisol			.014 (.008)	3.49	.015 (.013)	1.43	.015 (.008)	3.75*	.017 (.013)	1.60
Cortisol*Sex			**-.033 (.011)**	**9.66****	**-.052 (.018)**	**8.17****	**-.030 (.012)**	**6.14***	**-.052 (.018)**	**7.96****
Maternal Distress	.002 (.098)	.00					.012 (.098)	.02	-.010 (.099)	.01
Distress*Sex	**.328 (.153)**	**4.61***					**.297 (.155)**	**3.68†**	**.324 (.152)**	**4.56***
Cortisol*Distress							-.036 (.066)	.30	.025 (.118)	.05
Cortisol*Distress*Sex							**.056 (.109)**	**.26**	**-.029 (.161)**	**.03**
Quadratic Slope	-.321 (.052)	38.38***	-.338 (.048)	48.97***	-.317 (.051)	38.22***	-.316 (.051)	37.73***	-.323 (.052)	38.76***
Linear slope	.223 (.035)	39.39***	.239 (.036)	44.11***	.220 (.035)	39.36***	.230 (.035)	42.34***	.226 (.036)	40.57***

Infant Sex	.018 (.029)	.39	.022 (.033)	.42	.023 (.029)	.62	.007 (.029)	.06	.016 (.029)	.32
Maternal Cortisol			-.031 (.014)	4.74*	-.043 (.021)	4.23*	-.035 (.013)	7.56**	-.049 (.022)	5.17*
Cortisol*Sex			**.056 (.019)**	**7.88****	**.059 (.029)**	**4.00***	**.043 (.019)**	**4.58***	**.063(.030)**	**4.45***
Maternal Distress	-.013 (.150)	.01					-.036 (.149)	.06	-.015 (.152)	.01
Distress*Sex	**-.161 (.244)**	**.44**					**-.192 (.247)**	**.60**	**-.160 (.245)**	**.42**
Cortisol*Distress							.128 (.106)	1.45	.107 (.189)	.32
Cortisol*Distress*Sex							**-.377 (.177)**	**4.54***	**-.036 (.260)**	**.02**

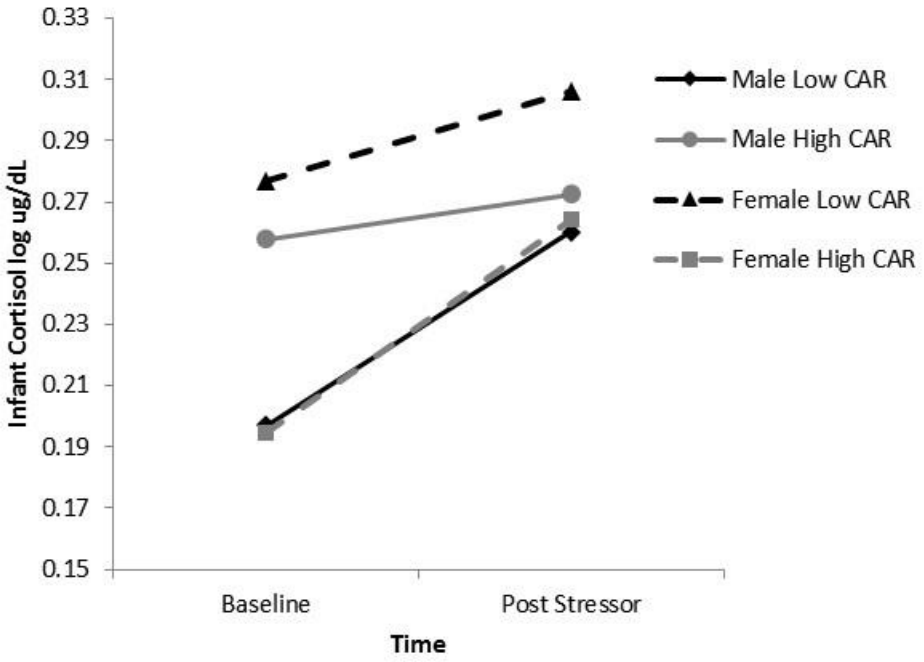
Note: † $p < .10$; * $p < .05$; ** $p < .01$; *** $p < .001$. GA= gestational age; CAR = cortisol awaking response; T1 = 14.9 weeks GA. Sex was centered at males. Bolded results are for the sex by prenatal exposure interactions. All covariates were centered at the sample mean with the exception of infant negative affect which was centered at 'no negative affect'.

Figure 1. Sex differences in infant cortisol reactivity as a function of prenatal distress exposure



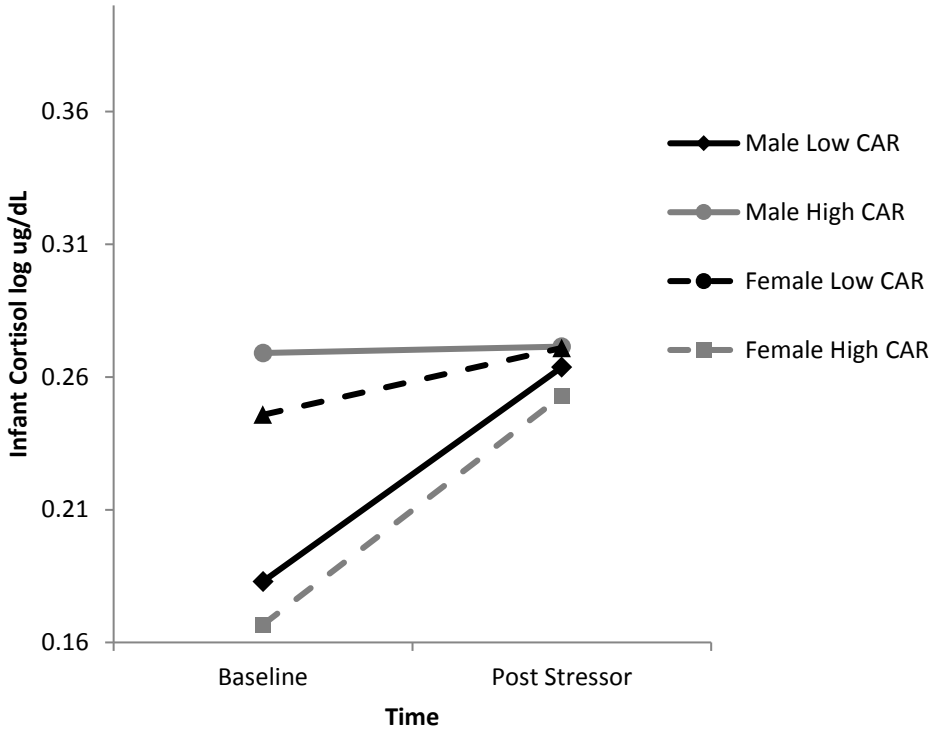
Note: Maternal psychological distress was modeled as a continuous variable but for illustration purposes we present it at the mean of the upper and lower quartiles to represent high and low distress, respectively. Post stressor is 20 minutes after the blood draw. The high and low distress lines for males are overlapping.

Figure 2.Infant cortisol reactivity as a function of sex and maternal CAR T1



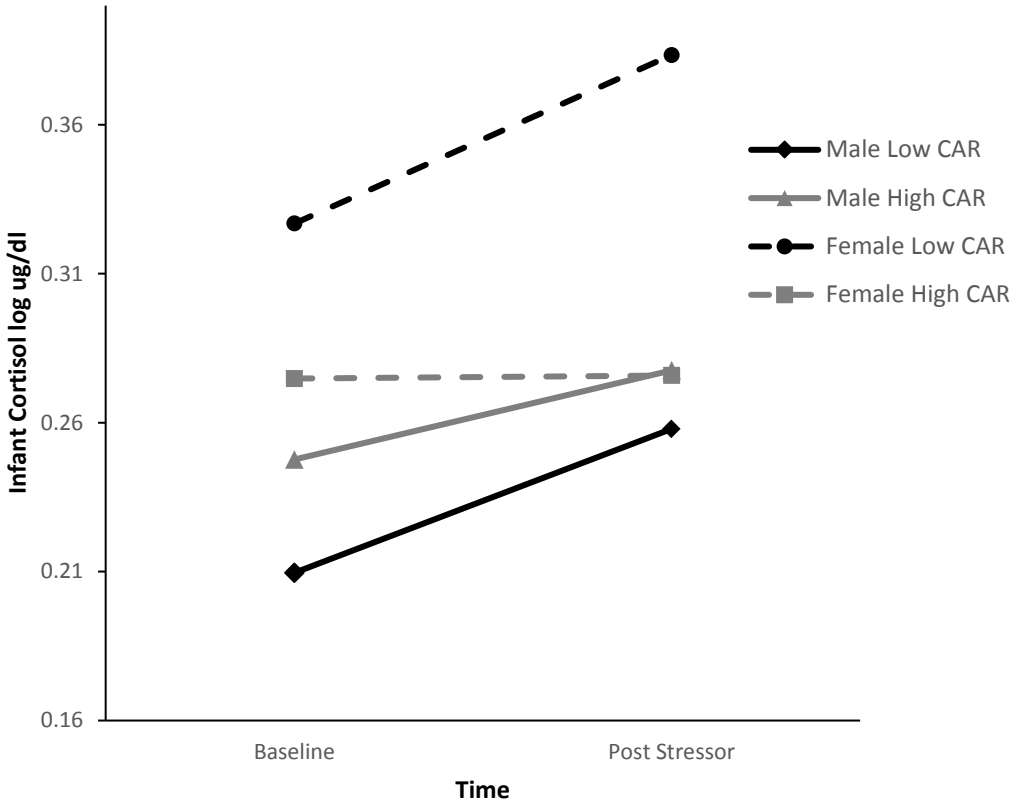
Note: CAR T1 = maternal cortisol awakening response in early pregnancy. CAR T1 was modeled as a continuous variable but for illustration purposes we present it at the means of the upper and lower quartiles to represent high and low CAR, respectively. Post stressor is 20 minutes after the blood draw.

Figure 3. Sex differences in the effects of maternal CAR T1 on infant stress reactivity at low prenatal distress exposure



Note: CAR T1 = maternal cortisol awakening response in early pregnancy; low prenatal distress = mean of the lower quartile. CAR T1 was modeled as a continuous variable but for illustration purposes we present it at the mean of the upper and lower quartiles to represent high and low CAR T1, respectively. Post stressor is 20 minutes after the blood draw.

Figure 4. Sex differences in the effects of maternal CAR T1 on infant stress reactivity at high prenatal distress exposure



Note: CAR T1 = maternal cortisol awakening response in early pregnancy; high prenatal distress = mean of the upper quartile. CAR T1 was modeled as a continuous variable but for illustration purposes we present it at the mean of the upper and lower quartiles to represent high and low CAR T1, respectively. Post stressor is 20 minutes after the blood draw.