Appendix S1 - SUPPLEMENTARY MATERIALS

SUPPLEMENTARY METHODS

Maternal Psychological Assessment

Antenatal depressive symptoms were evaluated through the Italian version (Benvenuti, Ferrara, Niccolai, Valoriani, & Cox, 1999) of the 10-items Edinburgh Postnatal Depression Scale (EPDS; Cox et al., 1987). The EPDS was originally designed to identify depressive symptoms in the postnatal period, but Murray and Cox (Murray & Cox, 1990) validated it also for pregnancy. The Italian version of this questionnaire showed an internal consistency of 0.79, a sensitivity of 0.61 and a specificity of 0.95 associated with a cutoff of 10 (Benvenuti et al., 1999).

Antenatal anxiety symptoms were evaluated through the Italian version (Pedrabissi & Santinello, 1989) of the 20-item state anxiety subscale of the State-Trait Anxiety Inventory (STAI) (Spielberger, Gorsuch, Lushene, Vagg, & Jacobs, 1970), a well-validated self-report questionnaire, which assesses anxiety symptoms that were experienced in the last few days. The Italian version of the STAI shows satisfactory values of internal consistency (0.93) (Pedrabissi and Santinello, 1989), sensitivity (0.81) and specificity (0.80) also for the perinatal period (Meades & Ayers, 2011).

Maternal Biological Assessment

Blood collection and assay. Maternal blood samples were obtained in a morning session at the hospitals between 34-36 weeks of gestation. After venipuncture, all samples were kept at +4° until they reached the Biological Lab of the Scientific Institute IRCCS Eugenio Medea where serum was centrifuged, aliquoted, and stored at −80 °C. Serum Interleukin-6 (IL-6) and C-Reactive Protein (CRP) concentrations were assayed in duplicate by using Quantikine High Sensitivity ELISA kits (R&D Systems Europe, LTD) at LaboSpace in Milan according to the instructions of the manufacturer. Intra- assay coefficient of variation (CV) was <6% for IL-6 and <3% for CRP, inter-assay CV was<10% for both markers.

Saliva collection and assay. Women collected six whole unstimulated saliva samples by passive drool as generally recommended (Granger et al., 2007). Samples were collected on two

consecutive days immediately upon awakening, 30 minutes after awakening and before going to bed in order to evaluate cortisol diurnal pattern. The times of saliva collection throughout the day were chosen as they are thought to provide an index of cortisol diurnal pattern (O'Donnell et al., 2013), while minimizing inconvenience for participants. Participants were carefully instructed to collect saliva and were given a saliva sampling pack which included six 2.0 mL colour-coded polypropylene cryovials, six shorten plastic straws, instructions and a diary where they were asked to report time of saliva collection. Participants were asked to refrain from eating, drinking (except for water), tooth-brushing and exercising in the 30 minutes before collection and eating a major meal in the hour before the evening sample. Pre-scheduled phone calls were performed by the researchers to remind women to perform saliva collections. All samples were kept on ice until reaching the Biology Lab of the Scientific Institute IRCCS Eugenio Medea where they were stored at -80°. All women provided completed saliva samples. The mean time from the collection upon awakening and the collection 30 minutes after was 30.82 minutes on antenatal day 1 (SD=3.64, range: 20.00-60.00) and 31.44 minutes on antenatal day 2 (SD=6.81, range: 20.00-90.00). All samples from one woman were run in the same assay to minimize method variability. On day of assay, the saliva samples were thawed, vortexed and centrifuged at 1500 x g for 15 minutes in order to remove mucins. Samples were assayed for cortisol in duplicate using a competitive high sensitivity enzyme immunoassay kit (Expanded Range High Sensitivity Cortisol EIA Kit, Salimetrics) specifically designed and validated for the quantitative measurement of salivary cortisol. Salivary cortisol assays were run in duplicate, except for 2 samples with minimal volume. Average intra- and inter-assay coefficients were <6% and <8%, respectively. Salivary alpha amylase (sAA) was assayed at the Salimetrics Centre of Excellence testing lab at Anglia Ruskin University, using a kinetic enzyme assay kit (Salimetrics α-Amylase Kinetic Enzyme Assay Kit). A random 10% of the sAA assays were run in duplicate to confirm reliability. The intra-assay coefficient of variation was <3%. Results were computed in µg/dl.

Infant assessment

Infant salivary cortisol. Three saliva samples were collected in the waiting room

immediately before (baseline) and 20 and 40 minutes after the 3-months routine inoculation visit using a specifically designed swab (SalivaBio Infant's Swab, Salimetrics). The times of saliva collection were selected in order to capture the peak cortisol response to the painful stressor (Davis et al., 2004; Gunnar, Talge, & Herrera, 2009). The inoculation is a widely employed physical stressor that is known to reliably activate the HPA axis in infants younger than 6 months of age (Jansen et al., 2009; Gunnar et al., 2009). Mothers were asked to arrive at the clinic 10 minutes before the time of their appointment, so there were at least 10 minutes between the arrival at the clinic and the baseline sample. While we cannot exclude that the first salivary sample might reflect infant's response to the new environment, baseline cortisol levels were significantly lower than cortisol levels collected after 20 and 40 minutes from the inoculation (ps<.001) thus suggesting that the pre-stressor cortisol sample provides a valid reference level for the activity of the HPA axis before the stressful condition. For the inoculation procedure, the infant was undressed, laid down and two injections, respectively, for the hexavalent and pneumococcal vaccine, were administered in the infants' thigh. Then the infant was given to the mother, who was free to soothe the baby. Mean duration of the shot was 1.06 minutes (SD=00.30). Only one parent (in the current study all mothers) was allowed to enter the medical office where the inoculation was performed. All the inoculations were performed in the morning, with the exception of 4 infants who were examined at 2 pm. Time of the day was examined as a covariate in the analyses. As milk in saliva can interfere with the cortisol assay (Granger et al., 2007; Magnano et al,. 1989), mothers were invited to feed the infants before leaving home. Thus, infants were not fed in the 30 minutes before the baseline saliva collection (mean time from last feeding=102.99 minutes, SD=54.60). However, 7 and 3 infants were breastfed, respectively, before the post-20 and post-40 saliva collections. In these cases, infants' mouths were gently swabbed with a wet sterile gauze prior to sample collection, in order to remove residue from milk. Infants' feeding before the saliva collection was examined as a covariate in the analyses and was not associated with infants' post-20 and post-40 cortisol levels (ps>.17). No use of medication was reported in the current sample, with the exception of one infant who was using a non-steroid topic cream (i.e. clotrimazole) for a vaginal mycosis. However, most infants were taking nutritional supplements. Specifically, 81 infants were taking D vitamin, 12 were

taking lactic ferments and 8 homeopathic products for colic. Use of nutritional supplements was examined as a possible confounder of the association between maternal cortisol and infant cortisol. Seventy-eight infants had complete cortisol data, whereas one (N=14) or two (N=2) samples were missing for 16 infants due to insufficient saliva volume. Infants with complete or partial data did not differ on any maternal, infants or situational variables (all ps>.10). Saliva samples were stored at -80º until assayed according to the same procedure described for maternal cortisol. All samples were run in duplicates (except for 8 samples with minimal saliva volume). The average intra- and inter-assay coefficients of variance were below 7% and 10%, respectively.

SUPPLEMENTARY ANALYSES

Supplementary analyses tested whether results could be replicated when wakening measures, rather than AUCg, were employed. As wakening measures are employed in the formula for the computation of AUCg, the two measures are related. The unadjusted Pearson correlation between cortisol wakening levels and AUCg was r=.39 p<.001, whereas between sAA wakening levels and AUCg was r=.60 p<.001. HLM analyses including waking measures rather than AUCg levels yielded comparable findings. Specifically, we reported a significant three-way interaction among maternal antenatal cortisol wakening levels, EA and both the linear (Estimate=-0.012, SE=0.005 p=.03) and quadratic (Estimate=0.00 SE=0.00 p=.03) time slopes on infant cortisol levels, while adjusting for gender, maternal age and postnatal depression. The interaction was in the same direction as that detected for maternal cortisol AUCg. Specifically, at higher levels of maternal antenatal wakening cortisol (+1SD), there was a significant association between maternal EA and the linear (Estimate=-0.009 SE=0.004 p=.02) and quadratic (Estimate=0.000 SE=0.000 p=.02) slopes of infant cortisol response with lower levels of maternal EA being associated with greater cortisol reactivity to the inoculation. In addition, at higher level of maternal EA (+1SD), there were no differences in infants' cortisol response to the inoculation depending on levels of antenatal maternal cortisol (ps=.14-15), while at lower levels of maternal EA (-1SD), infants antenatally exposed to higher maternal cortisol levels showed a marginally significantly steeper

linear and quadratic slopes (ps=.06). The overall improvement of the model fit over the baseline model was significant (deviance difference (9)=123.28, p<.001).

In contrast, consistently with findings on sAA AUCg, antenatal sAA wakening levels were not significantly associated with infant cortisol response either independently or in interaction with EA scores (ps>.70).

REFERENCES

Benvenuti, P., Ferrara, M., Niccolai, C., Valoriani, V., & Cox, J. L. (1999). The Edinburgh Postnatal Depression Scale: validation for an Italian sample. *Journal of Affective Disorders*, *53*(2), 137–141.

Cox, J. L., Holden, J. M., & Sagovsky, R. (1987). Detection of postnatal depression . Development of the 10-item Edinburgh Postnatal Depression Scale Detection of Postnatal Depression Development of the 10-item Edinburgh Postnatal Depression Scale. *Br J Psychiatry*, *150*, 782–786.

Davis, E. P., Townsend, E. L., Gunnar, M. R., Georgieff, M. K., Guiang, S. F., Ciffuentes, R. F., & Lussky, R. C. (2004). Effects of prenatal betamethasone exposure on regulation of stress physiology in healthy premature infants. *Psychoneuroendocrinology*, *29*(8), 1028–1036.

Granger, D. A., Kivlighan, K. T., Fortunato, C., Harmon, A. G., Hibel, L. C., Schwartz, E. B., & Whembolua, G. L. (2007). Integration of salivary biomarkers into developmental and behaviorally-oriented research: Problems and solutions for collecting specimens. *Physiology and Behavior*, *92*(4), 583–590.

Gunnar, M. R., Talge, N. M., & Herrera, A. (2009). Stressor paradigms in developmental studies: What does and does not work to produce mean increases in salivary cortisol. *Psychoneuroendocrinology*, *34*(7), 953–967.

Jansen, J., Beijers, R., Riksen-Walraven, M., & de Weerth, C. (2010). Cortisol reactivity in young infants. *Psychoneuroendocrinology*, 35, 329-338.

Magnano, C. L., Diamond, E. J., & Gardner, J. M. (1989). Use of salivary cortisol measurements in young infants: a note of caution. *Child development*, 60, 1099-1101.

Meades, R., & Ayers, S. (2011). Anxiety measures validated in perinatal populations: a systematic review. *Journal of Affective Disorders*, *133*(1–2), 1–15. .

Murray, D., & Cox, J. L. (1990). Screening for depression during pregnancy with the edinburgh depression scale (EDDS). *Journal of Reproductive and Infant Psychology*, *8*(2), 99–107.

O'Donnell, K. J., Glover, V., Jenkins, J., Browne, D., Ben-Shlomo, Y., Golding, J., & O'Connor, T. G. (2013). Prenatal maternal mood is associated with altered diurnal cortisol in adolescence. *Psychoneuroendocrinology*, *38*(9), 1630–1638.

Pedrabissi, L., & Santinello, M. (1989). Inventario per l'ansia di «Stato» e di «Tratto»: nuova versione italiana dello STAI Forma Y: Manuale. *Firenze: Organizzazioni Speciali*, *44*. JOUR.

Spielberger, C. D., Gorsuch, R. L., Lushene, R. E., Vagg, P. R., & Jacobs, G. A. (1970). State-trait anxiety inventory. Palo Alto. CA: consulting psychologists press.